Impact of target binding kinetics on in vivo drug efficacy: $k_{\text{off}}$, $k_{\text{on}}$ and …rebinding.


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Summary

Background and Purpose
Optimal drug therapy often requires continuing high levels of target occupancy. Besides the traditional pharmacokinetic contribution thereto, target binding kinetics is increasingly considered to play an important role as well. While most attention has been focused on the dissociation rate of the complex, recent reports expressed doubt about the unreserved translatability of this...
pharmacodynamic property into clinical efficacy. “Micro”-pharmacokinetic mechanisms like drug rebinding and partitioning into the cell membrane may constitute a potential fix.

Experimental approach.
Simulations based on solving differential equations.

Key results
Based on a selected range of association and dissociation rate constants, $k_{on}$ and $k_{off}$, and rebinding potencies of the drugs as variables, we have presently simulated their impact on the temporal \textit{in vivo} occupancy profile of their targets after one or multiple repetitive dosings.

Conclusions and implications
Most strikingly, the simulations show that, when rebinding is also taken into account, increasing $k_{on}$ may produce closely the same outcome as decreasing $k_{off}$ when dosing is performed in accordance with the therapeutically most relevant constant $[L_{max}]/K_D$ ratio paradigm. Also, under certain conditions, rebinding may produce closely the same outcome as invoking slow diffusion of the drug between the plasma- and a target-containing “effect” compartments.

Although the present simulations should only be regarded to constitute “proof of principle”, the findings may help pharmacologists and medicinal chemists to device \textit{ex-vivo} and \textit{in vitro} binding kinetic assays that are more relevant and translatable to \textit{in vivo} settings.

Keywords
Clinical efficacy
Compartment models
Drug binding kinetics
\textit{In vivo}
Pharmacodynamics
Pharmacokinetics
Rebinding
Residence time
Simulations
Abbreviations

3D: three-dimensional.
$k_{on}, k_{off}$: second- and first order rate constants for association and dissociation
$K_D$: Equilibrium dissociation constant
$k_a, k_e$: first order rate constants for the inflow and the elimination/clearance of the drug
$k_{eo}$: first order rate constant for the drug’s equilibration between the plasma and effect compartments
$[L], [L_e]$: concentration of drug in plasma (with $[L]$ at given time point) and in tissue “effect” compartment (with $[L_e]$ at given time point)
$k_{on}[L]$ (or $[L_e]$ for 2-compartment model): the rate coefficient that refers to the actual formation of new RL complexes at any time point
$[L_{max}] = C_{max}$ in pharmacokinetics: maximal concentration of drug near its target.
$[RL], [RL_{max}], [RL_{max}]_{eq}$: Concentration of drug-target complex, its maximal value after dosing and theoretical maximal value in case of instant equilibrium binding.
AUC: Area under the curve; corresponding here to the average occupancy of the target over a 24 h period.
$\text{Diss } t_{1/2}$: dissociation half-life ($= 0.69/ k_{off}$).

Table of links:

Ligands
Candesartan
Diprenorphine
N-methyl scopolamine
Olmesartan
Raclopride
Rimonabant
SCH 23390
Telmisartan
Spiiperone
1. Introduction.

Drug candidates have traditionally been optimized in terms of efficacy and potency (i.e. pharmacodynamic, PD, properties) while the duration of their in vivo pharmacological activity was linked to how their free concentration changes over time (i.e. a pharmacokinetic, PK, property). Except for irreversible/covalent binding mechanisms, the lifetime/residence time of drug-target complexes (also a pharmacodynamic property, Copeland 2006) was deemed to be too short to significantly contribute thereto. Accordingly, only a limited number of studies (e.g. Leysen and Gommeren, 1987; Vanderheyden et al., 2000) paid attention to binding kinetics until its importance was highlighted by the seminal reviews by Swinney (2004) and Copeland (2006). Their viewpoint is now corroborated by an increasing amount of observations (e.g. Bradshaw et al., 2015). Also, an important theoretical argument in favor of moving from binding affinity to binding kinetics is that, while drug concentrations are kept constant in in vitro experiments (so that equilibrium binding can be reached after a sufficiently long incubation time), this is not the case in the human body since it is an “open system” in where the concentration of free drug changes over time (Copeland, 2016).

The role of binding kinetics has become increasingly recognized and is now covered in many review articles and even in a recent book (Keserü and Swinney, 2015). Most attention therein has been focused on slow drug dissociation (i.e. a low dissociation rate constant, $k_{off}$) since this parameter is widely accepted to represent a key property of many marketed drugs. In this respect, simulations revealed that the clinical action of a drug lasts longer if it dissociates slower from its target than its in vivo PK elimination (Vauquelin and Van Liefde, 2006; Tummino and Copeland, 2008; Lu and Tonge, 2012; Dahl and Akerud, 2013). Yet, this focus on $k_{off}$ may be too restricted. First, some authors have also drawn attention to the utility of fast drug association (i.e. a high association rate constant, $k_{on}$) in clinical therapy (Yin et al., 2013, Schoop and Dey, 2015). More disturbingly, Dahl and Akerud (2013) recently even expressed concern about the relevance of including $k_{off}$ measurements in lead optimization programmes. Indeed, they found that the dissociation of many drugs and drug candidates from their target proceeds faster than their in vivo PK elimination, not slower. Based thereon, they concluded that PK usually prevails over binding kinetics.

In compliance with “Occam’s razor” principle, this conclusion is based on the simplest PD and PK models according to which drug-target interactions are represented as a reversible
single-step bimolecular process and PK elimination rates rely on drug concentrations in the
plasma, not in the vicinity of the target itself (Figure 1A). As a potential alternative
explanation for why a drug’s therapeutic effect often lags behind its plasma concentration, a
more complex “two-compartment” PK model with slow equilibration of the drug between the
plasma compartment and a hypothetical target- bearing “effect compartment” has already
been introduced some time ago (Holford and Scheiner, 1982) (Figure 1A). To illustrate this
lag, the drug’s effect is often represented as a function of its plasma concentration (Danhof et
al., 2008; Gabrielsson et al., 2009) where it gives rise to a counterclockwise hysteresis loop
with a depressed effect when the plasma concentration initially increases and an uplifted
effect when the concentration subsequently declines. With respect to the PD models, multi-
step “induced fit”-type binding mechanisms are now rather considered to play a key role for
achieving high affinity and clinical efficacy of many drugs (Copeland, 2016). Yet, despite of
this increased complexity, radioligand wash-out experiments (Figure 1B and Vauquelin,
2012) most often yield mono-exponential dissociation curves so that the overall dissociation
process can still be satisfactorily described by a “macroscopic” $k_{\text{off}}$ (Neubig et al., 2003;
Tummino and Copeland, 2008; Vauquelin et al., 2016). Regardless of the binding
mechanism, it is of note that such in vitro experiments still essentially focus on obtaining
“genuine” $k_{\text{off}}$ values and to this end, an excess of unlabelled competing ligand has been
routinely added to the wash-out medium to prevent “rebinding” of the radioligand (Figure
1B).

Since rebinding has already been covered in previous review articles (Vauquelin and
Charlton 2010; Vauquelin, 2010, 2015; Swinney et al., 2015) it is only its most essential
characteristics that are mentioned below and illustrated in Figure 1B. Its initial definition
referred to the establishment of a new mass-action type equilibrium binding between free
targets and freshly dissociated drug molecules, implying that those are able to disperse
rapidly all over the wash-out medium. However, subsequent research in different life sciences
disciplines led to a distinct conclusion, i.e. that it is a highly localized process that takes place
at the (sub)microscopic scale because of the presence of obstacles which hinder free 3D
diffusion of drug molecules away from their targets along with a high local density of those
targets. Those morphological properties prompt freshly dissociated drug molecules to go
through multiple encounters with their initial target and/or targets nearby before drifting
further away (Figure 1B). This will result in prolonged “apparent” target occupancy (Figure
1B). The morphological complexity of intact cells and tissues suggests that such “hindered-
diffusion” related rebinding is commonplace in vivo. Indeed, the cell represents a confined space (Copeland, 2016) and, in intact tissues, receptors can be concentrated in microdomains of the cell membrane (Pike, 2003) and also most often face cavities with little convective stirring like synapses and interstitial spaces. Early on, ex vivo experiments have already highlighted the physiological relevance of drug rebinding and special attention was also drawn to the need to use “still living” brain slices in order to observe this phenomenon (Perry et al., 1980; Sadée et al., 1982; Frost and Wagner, 1984; Gifford et al, 1989). Because they mimic the complexity of intact tissues to a reasonable extent, confluent plated cell monolayers can be used as surrogate model systems to study rebinding phenomena (Spivak et al., 2006). Using such recombinant cell monolayers, rebinding of several radiolabelled antagonists to their receptors has already been documented (reviewed in Vauquelin and Charlton, 2010). Interestingly, the radioligands that we tested in the thereto-dedicated wash-out experiments did not all experience rebinding to the same extent. This disparity is clearly illustrated by the two examples shown in Figure 2 of the Supplementary Information section and commented upon in the Discussion. Hence, although rebinding is considered to constitute a nuisance when the purpose is to determine genuine dissociation rates of a drug in e.g. radioligand wash-out experiments (Figure 1B), it may be regarded to represent a missing link between the measured $k_{off}$ values in vitro and the duration of the drug’s therapeutic effect in vivo (Zhang, 2015).

Although the potential contribution of hindered diffusion- based rebinding to longer-lasting target occupancy in vivo was already succinctly addressed elsewhere (Vauquelin, 2010, 2015; Swinney et al., 2015) the present study provides for the first time an extended, systematic evaluation thereof. The present simulations, in where administration/dosing of the drug is performed daily and in accordance with the therapeutically most relevant paradigm (Figure 1C), reveal that invoking rebinding may prolong its therapeutic effect in closely the same way as by decreasing $k_{off}$ (i.e. increasing the lifetime of the drug-target complex itself) as well as by invoking slow diffusion of the drug between the plasma- and a target-containing “effect” compartment. Moreover, whereas review articles about binding kinetics essentially focus on $k_{off}$, the present findings also suggest that the association rate, $k_{on}$, merits closer attention in drug design because of its important contribution to robust rebinding.
2. Definitions, Paradigms and Methods.

2.1. Equations for a “closed system”.
A closed system refers here to a system in which the total drug and target concentrations remain steady with time. We will here focus on the simplest mechanism of drug-target complex, LR, formation; i.e. a reversible bimolecular interaction that obeys the law of mass-action. The equilibrium dissociation constant, $K_D = k_{\text{off}}/k_{\text{on}}$ (where $k_{\text{on}}$ is the second-order association rate constant and $k_{\text{off}}$ the first-order dissociation rate constant), constitutes a traditional metrics for the drug’s potency (Hulme, and Trevethick, 2010). The differential equations 1 and 2 (Table 1) were consecutively solved over very small time intervals to simulate how $[R]$ and $[RL]$ change over time for such closed system (Vauquelin et al., 2001).

2.2. Equations for a single-compartment in vivo setting for different dosing paradigms.
Living organisms are “open systems” wherein, after a single dosing, the concentration of free drug near the target, $[L]$, first increases and then declines. In the simplest “one compartment” body model (Figure 1A), this bell-shaped pattern can be described by the Bateman function (Garrett, 1994): i.e.

$$[L] = f.\left(\frac{k_a}{k_a-k_e}\right).\left(e^{-k_e.t} - e^{-k_a.t}\right)$$

The first order rate constants, $k_a$ and $k_e$, correspond to the inflow and the elimination/clearance of the drug, respectively. They remain the same throughout this study (i.e. 0.0115 and 0.00575 min$^{-1}$, for $t_{1/2} = 60$ and 120 min). The maximal concentration of free drug near the target, $[L_{\text{max}}]$ (denoted as $C_{\text{max}}$ in pharmacokinetics), is attained 120 min after dosing and, since only the amplitude of those plots differs; $[L_{\text{max}}]$ and the concentration of free drug at each time point, $[L]$, will thus vary alike. The parameter, ‘$f$’, in the Bateman function accounts for the drug’s dose, bioavailability and volume of distribution. For the present simulations, $f$ was set to obtain a value of $[L_{\text{max}}]$ that allows the desired amount of maximal target occupancy, $[RL_{\text{max}}]_{\text{eq}}$, under instant equilibrium conditions (i.e. via $[L_{\text{max}}] = K_D/([R_{\text{tot}}]/[RL_{\text{max}}]_{\text{eq}} -1)$. Of note is that the value of $[RL_{\text{max}}]_{\text{eq}}$ as well as its ability to be attained at the same moment as $[L_{\text{max}}]$ are only theoretical. In practice, the actual “peak binding”, $[RL_{\text{max}}]$, will be less and will be attained later on because the association and dissociation events are not instantaneous.
Simulations that take account of binding kinetics to mimic how [L] and [RL] change over time have already been performed. In some of the comparative studies [L_{\text{max}}] was kept the same irrespective of their binding kinetic parameters and K_D while, in others, it was the [L_{\text{max}}]/K_D ratio that remained the same for all the drugs (Figure 1C). As illustrated in the Results section, the dosing paradigm has an important impact on how k_{on} and k_{off} affect how [RL] evolves over time. Equations 3 and 4 in Table 1 apply when dosing complies with the constant [L_{\text{max}}] paradigm (i.e. mimicking an in vivo situation in where the dose is kept the same irrespective of the ligand’s K_D) and equations 5 to 7 in Table 1 apply when dosing complies with the constant [L_{\text{max}}]/K_D ratio paradigm (i.e. mimicking an in vivo situation in where the dose varies in par with the K_D of each drug in question). For the first paradigm, changing k_{on} or k_{off} will affect the drug’s K_D but not [L_{\text{max}}] (and thus also not [L]_t). Accordingly, changing k_{on} triggers an equivalent change of the k_{on}.[L]_t product; i.e. the rate coefficient that refers to the actual formation of new RL complexes at any time point. For the second paradigm, changing k_{on} will changes K_D, [L_{\text{max}}] and [L]_t in the opposite way. Consequently, the k_{on}.[L]_t product remains unchanged. On the other hand, changing k_{off} now triggers an alike change of the k_{on}.[L]_t product.

2.3. Equations for a two-compartment in vivo setting.

A two-compartment model with slow equilibration of the drug between the plasma compartment and a hypothetical target-bearing “effect compartment” has been introduced (Holford and Scheiner, 1982) to explain why a drug’s therapeutic effect often lags behind its plasma concentration (Figure 1A). The rate by which the drug transfers between both compartments is here governed by a single constant, k_{eq}. The Bateman function still accounts for concentration of free drug in the plasma compartment, [L], while its concentration in the effect compartment, [L_e], is calculated by solving equation 8 in Table 1. Replacing [L]_t by [L_e]_t in equations 5 to 7 allows the temporal evolution of [RL] to be simulated.

2.4. Equations for rebinding.

The equations that govern hindered diffusion-related rebinding have already been extensively documented upon in a previous review article (Vauquelin and Charlton, 2010). In short (more explicit information is provided in the legend of Figure 1B), k_{on} is replaced by the ‘effective’ forward rate coefficient (k_f = k_{on}/(1 + k_{on}[R]/k) and k_{off} by the ‘effective’ reverse rate coefficient (k_r = k_{off}/(1 + k_{on}[R]/k). The robustness of rebinding at any level of target occupancy, k_{on}.[R]/k, is maximal when all the targets are free, i.e. when [R] = [R_{\text{tot}}]. This
maximal value will be referred to as the “rebinding factor”. \( k_{on}[R]/k \) Can then be calculated by multiplying this rebinding factor by \([R]/[R_{tot}]\). As shown in Figure 1 of the Supplementary Information section, \( k_f \) and \( k_r \) are lowest when all the targets are free and do gradually increase till they equal \( k_{on} \) and \( k_{off} \) when all the targets are occupied. Accordingly, rebinding is more prominent at low- than at high levels of target occupancy. To account for rebinding, the differential equations in Table 1 were appended as indicated.

2.5. Relevance of kinetic parameters and the rebinding factor.

With the chosen order rate constants for the inflow and the elimination/clearance of the drugs \( (k_a = 0.0115 \text{ min}^{-1} \) and \( k_e = 0.00575 \text{ min}^{-1} \)), the evolution of \([L]\) with time is very similar as in Dahl and Akerud (2013). Also, the chosen range of \( k_{on} \) (1.10\(^6\) to 1.10\(^8\) M\(^{-1}\).min\(^{-1}\)) and \( k_{off} \) values for the binding process applies to the majority of the drugs that were investigated by those authors (Figure 6 in their publication). In accordance with extrapolations from intact cell-based radioligand washout experiments (please see Figure 2 of the Supplementary Information section for extremes) the rebinding factor, \( k_{on}[R_{tot}]/k \), was allowed to vary between 3 to 100 in the present simulations.

3. Results.

3.1 Simulations without rebinding.

After a recapitulation about how \([RL]\) changes over time when \([L]\) decreases exponentially, subsequent simulations show how \([RL]\) evolves with time when the \([L]\) vs time plots adopt a bell-shaped pattern such as after \textit{in vivo} dosing. Besides the impact of the drug’s \( k_{on} \) and \( k_{off} \) thereon, attention is (for the first time) also paid to the different dosing paradigms that have previously been used for related previous studies.

For a simple reversible bimolecular binding mechanism, \([RL]\) will decline mono-exponentially with time when free ligand is abruptly removed or prevented to associate (Figure 2A). Simulated data in Figure 2B,C show that this no longer applies when \([L]\) also declines mono-exponentially. Here, even when the dissociation is very fast (i.e. for high \( k_{off} \)), \([RL]\) declines slower than the drug’s elimination because of the hyperbolic relationship between \([RL]\) and \([L]\) (Vauquelin and Charlton, 2010). An initial lag phase can be observed, especially when \([RL]\) is high at the start. In agreement with earlier findings (Vauquelin and Van Liefde, 2006; Tummino and Copeland 2008; Vauquelin and Charlton, 2010; Lu and
Tonge, 2010; Dahl and Akerud, 2013), Figure 2A-C also shows that slow dissociation only substantially prolongs the occupancy when L is eliminated more swiftly.

Simulations in where [L] varies with time according to the Bateman function, with a rapid increase followed by a slower decline such as after in vivo dosing (Figure 2D), are more relevant from the clinical perspective. In such studies, most attention has been focused on how \( k_{\text{off}} \) affects the evolution of \([RL]\) over time. Here also, it was noticed that \( k_{\text{off}} \) has to drop below \( k_e \) to produce longer-lasting occupancy (Tummino and Copeland 2008; Vauquelin, 2010, 2015; Dahl and Akerud, 2013). On the other hand, such studies also led Yin et al. (2013) to conclude that \( k_{\text{on}} \) also contributes to important pharmaceutical properties in “subtle but different ways”. Yet, only scant attention has hitherto been paid to the fact that such studies were not all based on the same dosing paradigm. In some, \([L_{\text{max}}]\) (and thus the dose) was kept the same irrespective of the drug’s \( K_D \) (Copeland, 2006; Tummino and Copeland 2008, 2008; Yin et al., 2013) while, in others, \([L_{\text{max}}]\) was allowed to vary in par with the \( K_D \) of each drug in question; i.e. the \([L_{\text{max}}]/K_D\) ratio was kept constant (Dahl and Akerud, 2013; Vauquelin, 2010, 2015). The next simulations aim to provide a better insight into the potential impact of these dosing paradigms on how \( k_{\text{on}} \) and \( k_{\text{off}} \) affect the occupancy pattern.

Simulations for the constant \([L_{\text{max}}]\) paradigm (in where the \([L]\) vs. time plot shown in Figure 2D applies to all the drugs tested) show that increasing \( k_{\text{on}} \) or decreasing \( k_{\text{off}} \) produces a quite comparable increase in peak occupancy (Figure 2E). This is to be expected because both actions decrease the drug’s \( K_D \). Yet, in line with the different impact of \( k_{\text{on}} \) and \( k_{\text{off}} \) on the equations that govern the occupancy profile (please see equations 3 and 4 in Table 1 and Section 2.3 for further explanation) changing \( k_{\text{on}} \) or \( k_{\text{off}} \) will not affect the occupancy profile in exactly the same way. Varying \( k_{\text{on}} \) has the largest impact on the initial ascending phase as shown by the already high levels of occupancy at early time points at high \( k_{\text{on}} \) (Figure 2F) and the delayed attainment of peak occupancy at low \( k_{\text{on}} \) (Figure 2E). Eventually, when peak occupancy only shows up when \([L]_t\) has already dropped substantially, its magnitude, \([RL_{\text{max}}]\), will also start to drop. On the other hand, the impact of \( k_{\text{off}} \) is more outspoken on the subsequent decline in target occupancy; this decline will become substantially slower when \( k_{\text{off}} \) drops below \( k_e \) (Figure 2E). Yet, a handicap of this dosing paradigm is that, by changing the theoretical peak occupancy, any change in \( k_{\text{on}} \) or \( k_{\text{off}} \) will also indirectly affect the rate of this decline (see also Figure 2B,C).
This latter source of interference is avoided when the simulations are based on the constant $[L_{\text{max}}]/K_D$ ratio paradigm. The influence of $k_{\text{on}}$ and $k_{\text{off}}$ on the occupancy profile becomes now also clearly distinct. All occupancy curves now overlap when only $k_{\text{on}}$ is allowed to vary (Figure 2G). As outlined in Section 2.3 and shown in equation 5 in Table 1, this invariance stems from the fact that any change of $k_{\text{on}}$ is now cancelled out by the inverse change of $[L]_t$, so that the $k_{\text{on}}[L]_t$ product remains unaffected. In contrast, only varying $k_{\text{off}}$ has an outspoken impact on the occupancy profile (Figure 2H). On the other hand, decreasing $k_{\text{off}}$ will not only prolong the occupancy when it drops below $k_c$ but it will eventually also sizably delay peak binding and lower $[RL_{\text{max}}]$. This stems from the fact that any change of $k_{\text{off}}$ also produces an equivalent change of the $k_{\text{on}}[L]_t$ product (equation 6 in Table 1). The different repercussions of decreasing $k_{\text{off}}$ will also act together to exacerbate counterclockwise hysteresis loop in the corresponding occupancy vs. $[L]$ representation (Figure 2I).

Interestingly, the occupancy curves remain exactly the same as those shown in Figure 2G when both $k_{\text{off}}$ and $k_{\text{on}}$ are changed alike to yield an iso-$K_D$ situation in simulations that are based on the constant $[L_{\text{max}}]$ paradigm (Figure 1C). Comparing equations 6 and 7 in Table 1 reveals that this similitude stems from the equivalent contribution of $k_{\text{on}}$ and $[L]_t$ to the $k_{\text{on}}[L]_t$ product.

3.2. Including rebinding in the simulations.

The next simulations compare the effect of “diffusion-limited” rebinding (please see Figure 1B for basic information) and $k_{\text{off}}$ on the occupancy profile under the in vivo dosing conditions the are most relevant one from the clinical perspective. To this end, we will from now on only focus on the on the constant $[L_{\text{max}}]/K_D$ ratio paradigm. Indeed, real-life dosing is still highly influenced by the drug’s potency (Dahl and Akerud, 2013). An additional advantage of this paradigm is that $k_{\text{on}}$ contributes to the robustness of rebinding but does not impact the occupancy profile by itself (Figure 2G). This greatly simplifies the re-evaluation about how $k_{\text{on}}$ affects the drug’s occupancy profile when rebinding is allowed to take place.

As stipulated in Section 2.3 and shown in Figure 1 of the Supplementary Information section, “diffusion-limited” rebinding will exert a maximal impact on the association and dissociation kinetics when all the targets are free and this will gradually decline till nil when the occupancy increases. As a convenient single metrics for the rebinding intensity, we will use its highest
value, \( k_{\text{on}}[R_{\text{tot}}]/k \), and refer to it as the “rebinding factor”. The rebinding intensity at any level of target occupancy can then be easily calculated by multiplying this factor by \([R]/[R_{\text{tot}}]\).

The simulations that are shown in Figure 3A-F compare the influence of this rebinding factor on the occupancy profile of drugs with fast (high \( k_{\text{off}} \), panel A) to very slow dissociation (low \( k_{\text{off}} \), panel F). In all instances, increasing the rebinding factor (here obligatorily by increasing \([R_{\text{tot}}]\) and/or decreasing \( k \) to deal with the fact that \( k_{\text{on}} \) is kept constant) will gradually prolong the occupancy and, alongside, delay peak binding and lower its magnitude. While barely perceptible at high \( k_{\text{off}} \), these phenomena become gradually more pronounced when \( k_{\text{off}} \) decreases. Interestingly, decreasing \( k_{\text{off}} \) or increasing the rebinding factor by the same amount results in quite similar occupancy profiles (more situations are shown in Figure 3 of the Supplementary Information section). This similarity is also evident when comparing the 24 h post-administration area under the curve (AUC) values. As shown in Figure 3G, the impact of decreasing the dissociation \( t_{1/2} \) (abscissa) on the AUC’s is effectively counteracted by increasing the rebinding factor (from red to blue) by the same amount or, in other words: rebinding allows the same effect to be obtained with faster dissociation.

Similar to the simulations without rebinding (Figure 2G), varying \( k_{\text{on}} \) does not affect the occupancy curve either if the rebinding factor is kept constant (Figure 3H). Here again, this can be explained by the ability of \([L]\) to cancel out any change of \( k_{\text{on}} \). Yet, it is reasonable to assume that the target’s concentration and microenvironment remain fixed in comparative in vivo studies so that the rebinding factor is proportional to \( k_{\text{on}} \). This should allow \( k_{\text{on}} \) to modify the occupancy profile via the intermediary of rebinding. Based on a drug with \( k_{\text{on}} = 1.10^{7} \text{ M}^{-1}\text{ min}^{-1} \), \( k_{\text{off}} = 6.9.10^{-3} \text{ min}^{-1} \) (diss \( t_{1/2} = 100 \text{ min} \)) and rebinding factor = 10 as median (Figure 3H), Figure 3I-L shows that changing \( k_{\text{on}} \) (open circles) or \( k_{\text{off}} \) (solid lines) by the same extent affects the occupancy profile in closely the same way. Taken together, while \( k_{\text{on}} \) has no effect on the occupancy profile without rebinding (Figure 2G), increasing a drug’s \( k_{\text{on}} \) will impact this profile in closely the same way as increasing \( k_{\text{off}} \) when rebinding is also taken into consideration. This situation is most likely to apply for tissues in living organisms.

3.3. Comparison of the impact of rebinding to that of the two-compartment PK model.

Slow equilibration of the drug between the plasma compartment and a hypothetical target containing “effect compartment” was initially invoked to explain the effect of a drug often lags behind its plasma concentration (Sheiner et al., 1979; Holford and Scheiner, 1982). The
next simulations reveal that rebinding may produce closely the same outcome for drugs that dissociate sufficiently slow.

Simulations in Figure 4 refer to a relatively slow dissociating drug (diss. $t_{1/2} = 100$ min). Its occupancy profile without rebinding or equilibration is shown as control (black curve) in occupancy vs time plots in Figure 4A-C and the corresponding occupancy vs plasma concentration, $[L]$, plots in Figure 4D-F. Although this drug already generates a moderate counterclockwise hysteresis by itself, introducing rebinding (red curves) or slow equilibration (governed by the rate constant, $k_{eo}$, blue curves) will both progressively exacerbate this pattern by decreasing peak occupancy as well as by delaying its attainment and prolonging the occupancy. For the sake of comparison, $k_{eo}$’s were chosen to yield closely the same peak occupancy as with rebinding in each panel. Although not strictly overlapping, the plots for rebinding and slow equilibration are quite comparable in each panel.

Such simulations were also carried out for a very fast dissociating drug (diss $t_{1/2} = 0.1$ min). To better compare the observations with those of the previous drug, the $k_{eo}$’s and rebinding factors were kept the same. Curves are here also in black for the control situation without rebinding or equilibration. Figure 5A,D depicts how the concentrations of free drug in the plasma, $[L]$ (black curves), and in the effect compartment, $[L_e]$ (colored curves) changes over time. For the control situation, the purely hyperbolic shape of the occupancy vs $[L]$ plot in Figure 5C,F is typical for equilibrium binding. Here again, introducing slow equilibration gives rise to counterclockwise hysteresis loop and progressively exacerbates this pattern by decreasing peak binding, delaying its attainment and prolonging the occupancy (Figure 5B,C). In contrast, no change in the temporal occupancy profile can be perceived when introducing increasing degrees of rebinding instead (Figure 5E). Also, the more sensitive occupancy vs $[L]$ representation reveals very little change, except for the highest rebinding factor and at very early time points only (Figure 5F). Taken together, while (under the presently examined conditions) rebinding and slow equilibration may impact the occupancy profile of a slow dissociating drug quite similarly (Figure 4), only the latter mechanism will be able to do so for a fast dissociating drug (Figure 5).

For the slow dissociating drug, a potential approach to discern between its rebinding and slow equilibration in *ex-vivo* radioligand binding experiments relies on the rationale (Vauquelin and Charlton, 2010; Vauquelin and Van Liefde, 2012) that it is only in case of rebinding that
adding an excess of competing drug in the wash-out medium should be able to enhance the “apparent” dissociation of the radioligand. This phenomenon is more clearly illustrated in Figure 4G-I. Setting \([L] = 0\) at any time after the dosing (to mimic removal of free radioligand and subsequent wash-out in the presence of an excess of competitor) yields indeed a faster decline in the occupancy than by setting \([L] = 0\) alone (to mimic removal of free radioligand only). Interestingly, such observations have already been made for real-life situations (Perry et al., 1980; Sadée et al., 1982; Frost and Wagner, 1984; Gifford et al, 1989).

3.4. Repetitive dosing and increasing the dose.

The previous simulations only dealt with a single dosing. The next simulations extend this exploration to repeated daily dosings for up to 8 days. Relevant curves are shown in Figure 6, their corresponding AUC’s for day 1 to 8 are shown in in Figure 7A-L and their peak-to-trough occupancy ratios at day 8 in Figure 7M-O (blue curve). For fast dissociation and low rebinding, trough occupancy is nearly insignificant so that the curves after consecutive dosings overlap (Figure 6A,B,D,G). The AUC’s remain constant and the peak-to-trough ratios remain equally high. Gradually decreasing \(k_{\text{off}}\) and/or increasing the rebinding factor will progressively break this pattern, first by enabling a moderate increase in peak occupancy on day 2 only (Figure 6C,E,J), then also on day 3 and so on. As shown in Figure 6I-L, peak occupancy still increases on day 8 for the slowest dissociation/highest rebinding combinations. This offers an at least partial compensation for the much-reduced peak binding of these latter drugs at day 1 (as also shown in Figure 3E,F). Also, their AUC’s (Figure 7I-L) will gradually increase during the ensuing days. Finally, the appreciably lower daily fluctuations in their target occupancy is also reflected by the smaller peak-to-trough ratios (Figure 7N,O).

The effect of increasing the daily dose (i.e. \([L_{\text{max}}]/K_D\) ratio) on the occupancy profile at day 1 is depicted for an extended range of \(k_{\text{off}}/\text{rebinding}\) combinations in Figure 3 of the Supplementary Information section. Taken together, increasing the dose will increase the AUC’s (Figure 7A-L) and decrease the peak-to-trough ratios (Figure 7M-O) for all the drug/rebinding combinations. Yet, the most notable consequence of this initiative resides in the earlier attainment of the maximal AUC’s.
Discussion

The present simulations are aimed to evaluate the impact of \( k_{\text{off}} \), \( k_{\text{on}} \) as well as hindered diffusion- based rebinding of a drug on how the occupancy of its target changes over time after \textit{in vivo} dosing. In this respect, the hitherto prevailing review articles on binding kinetics principally focused on \( k_{\text{off}} \). At odds therewith, open system- based simulations and earlier findings (Bairy and Wong, 2010) led Yin et al (2013) to conclude that \( k_{\text{off}} \) alone does not provide a complete picture and that a relatively fast association rate is needed to design a good competitive inhibitor (sic). Of note is that their simulations were based on the constant \([L_{\text{max}}]\) paradigm (see Section 2.2). While, the present simulations also show that \( k_{\text{on}} \) affects the occupancy profile under such dosing conditions (Figure 2E,F) this is no longer the case when dosing complies with the clinically more relevant constant \([L_{\text{max}}]/K_D\) ratio paradigm in where any change of \( k_{\text{on}} \) is compensated for by the dosing (Dahl and Akerud, 2013). In contrast, the impact of \( k_{\text{off}} \) on the occupancy profile becomes even more pronounced for this latter dosing paradigm (Figure 2G-I). Besides triggering longer-lasting target occupancy, decreasing \( k_{\text{off}} \) does eventually also delay the attainment of peak occupancy and even produce a decline thereof. These comparative simulations thus shed light on an important issue, namely that the impact of a drug’s binding kinetic properties on its occupancy profile needs to be appraised in light of the dosing paradigm that was used.

Further simulations that were based on the constant \([L_{\text{max}}]/K_D\) ratio dosing paradigm revealed that that allowing dissociated drugs to bind again to the same or to surrounding targets before drifting away (i.e. “rebinding”) does affect the occupancy profile in a quite similar (but not strictly identical, see section 2.4) way as by lowering \( k_{\text{off}} \) (Figures 3 and 6). Moreover, via the intermediary of such rebinding, increasing \( k_{\text{on}} \) becomes now also able to impact the occupancy profile in the closely the same way as decreasing \( k_{\text{off}} \). This aspect is especially interesting in light of the recent claim by Dahl and Akerud (2013) that many therapeutic drugs have a slower elimination- than dissociation rate, which implies that dissociation kinetics alone should not contribute the long-lasting therapeutic effect of these drugs. Yet, because increasing rebinding may act a surrogate for decreasing the drug’s \( k_{\text{off}} \), the potential limitation of such “ineffective” dissociation rate (and also if the target’s local concentration and microenvironment do not optimally contribute to rebinding), should be offset partially by very large \( k_{\text{on}} \) values. In this respect, although the largest \( k_{\text{on}} \) that was used for the present simulations is sufficient to embrace most of the drugs that were taken in consideration by
Dahl and Akerud (2013), this value is still about 100-fold less than what is permitted by the diffusion limit. In the same vein, as shown in Figure 2A,B (and also in Figure 3 of the Supplementary Information section for in vivo dosing), increasing the \([L_{\text{max}}]/k_{\text{off}}\) ratio could also prolong the clinical benefit of such “sub-optimal” drugs provided that this does not exacerbate potential detrimental side effects. Taken together, though alternative mechanisms like tissue accumulation and the formation of active metabolites may also contribute, the present simulations thus suggest that prominent rebinding (and if necessary a large \(k_{\text{on}}\)) may already be sufficient to reinstate the causal link between slow dissociation and the therapeutic efficacy of “border-line” drugs like the candesartan (dissociation \(t_{1/2}\) of about 2 h vs. elimination \(t_{1/2}\) from the plasma of about 3.5 h) (Fierens et al., 1999a; Delacretaz et al., 1995) and some of those that were listed by Dahl and Akerud (2013).

Interestingly, not all drugs seem to experience rebinding to the same extent. To deal with this issue, the rebinding factors (i.e. \(k_{\text{on}}[R_{\text{tot}}]/k\)) that were used for the present simulations (from 3 to 100) were chosen to embrace those that were extrapolated from intact cell- based radioligand washout experiments (for illustrative examples please see Figure 3 of the Supplementary Information section). Based on ex vivo wash-out experiments with \(^{3}\text{H}\)-diprenorphine, Perry et al., (1980) also reported a value of 6. We feel that it is hazardous to advance numerical values for \([R_{\text{tot}}]\) and \(k\). The likely heterogenous distribution of the targets at the sub-microscopic level, such as the accumulation of receptors in lipid rafts and other microdomains of the membrane (Pike, 2003), constitute an obstacle for advancing relevant values of \([R_{\text{tot}}]\). Similarly, \(k\) is likely to be a complex function since it not only depends on the drug’s local diffusion rate but also of the geometry of the target’s local micro-anatomic environment (Coombs and Goldstein, 2004). The reason for the relatively large variance between the rebinding intensity of distinct drugs is presently unknown and merits to be further looked into. In this respect, it is noteworthy that hydrophobic GPCR- binding radioligands like \(^{3}\text{H}\)-spiperone and \(^{3}\text{H}\)-taranabant experience more rebinding than those that display less non-specific binding to plated cells like \(^{3}\text{H}\)-candesartan, \(^{3}\text{H}\)-olmesartan, \(^{3}\text{H}\)-telmisartan, \(^{3}\text{H}\)-rimonabant and \(^{3}\text{H}\)-raclopride (Reviewed in Vauquelin and Charlton, 2010). Besides examining the drug-target interaction itself (e.g. the influence of ligand structure, target- mutation, expression level and micro-anatomic distribution), it should therefore also be of interest to evaluate the potential impact of membrane lipids as well as of the contiguous “unstirred water layer” (on e.g. the drug’s conformation and mode of approach to the target) on the robustness of rebinding (Sargent and Schwyzter, 1986; Abdiche
and Myszka, 2004; Szczuka et al., 2009; Vauquelin and Packeu, 2009; Fotakis et al., 2011; Loftsson, 2012).

Increasing the overall/macroscopic residence time by decreasing \( k_{off} \) and/or increasing rebinding may be unfit for certain therapeutic indications (Copeland, 2010, Núñez et al., 2012) since it will eventually go along with lower peak occupancy, delayed the attainment thereof and also reduce the average daily occupancy of the target (quantified by a lower post-dosing AUC). Yet, on the positive side, the occupancy will fluctuate less with time (quantified by a lower peak-to-through occupancy ratio). This may also bring about a higher peak occupancy and AUC after each consecutive dosing until those parameters eventually level off (Figures 6 and 7). This maximal AUC will exceed that of a fast-dissociating drug and, by administering a higher dose, this parameter increase even more and require less consecutive administrations to be attained (Figure 7).

The effect of an administered drug often lags behind its plasma concentration. From the early pharmacokinetic viewpoint in where target binding was supposed to reach rapid equilibrium, this delay was attributed to a slow equilibration of the drug between the plasma compartment and a hypothetical target-containing “effect compartment” within the tissue of interest (Holford and Scheiner, 1982; Derendorf and Meibohm, 1999; Danhof et al., 2008). Seen the now well-recognized impact of binding kinetics on the \( \text{in vivo} \) occupancy profile and the increasing awareness about the supplementary impact of rebinding thereon, it was of interest to compare the ability of slow equilibration and rebinding to delay the occupancy profile of different drugs. While only slow equilibration was efficient for the fast dissociating drug, moderate rebinding could already produce a comparable delay for the slow dissociating drug (Figures 4 and 5). These simulations raise the question whether slow equilibration between two compartments can be replaced by rebinding. Slow equilibration may still be important in solid tumors with long distances between the blood capillaries and the target cells and for drugs that slowly cross the blood-brain-barrier and cell membranes in general (Smith et al., 2010). On the other hand, the leaky pores in the blood capillaries in many other tissues allow a rapid equilibration between the drug’s concentration in the plasma and in the extracellular fluid that is in direct contact with major targets such as GPCRs and ion channels. In this situation, rebinding is likely to play a paramount role, as shown by the ability of unlabelled competitors to accelerate the release of e.g. \(^3\text{H}\)-N-methyl-scopolamine from isolated guinea...
pig atria and of \[^3\text{H}\]SCH 23390 from brain slices in which the blood-brain barrier is absent (Lullmann et al., 1988; Gifford et al., 1998)

In conclusion, Drug rebinding may be regarded to be a natural consequence of hindered 3D diffusion because of the morphological properties of our tissues and cells as well as of local target accumulation within cells or on the membranes thereof. Taking account of this phenomenon could therefore be of help to design \textit{in vitro} binding kinetic assays that are more relevant and translatable to \textit{in vivo} settings (Cusack et al., 2015; Zhang, 2015). Although the present simulations should only be regarded to constitute “proof of principle”, the obtained results suggest that $k_{\text{on}}$ merits more consideration in drug design because of its important contribution to robust rebinding. This parameter is presently often neglected/dismissed in the currently prevailing viewpoint about the link between a drug’s binding kinetics and its therapeutic efficiency. Incidentally, the observation that increasing $k_{\text{on}}$ and decreasing $k_{\text{off}}$ affect the occupancy profile in the closely the same way in the presence of rebinding (Figures 3 and 6), it is reasonable to deduce that a high affinity of the drug for its target (i.e., low $K_D$) has a positive impact on the duration of a drug’s \textit{in vivo} pharmacological activity.

**Author contributions.**

GV contributed to study design, simulations, writing, artwork.

**Conflict of interest.**

None.

**Supporting information**

Figure S1: Simulated effect of rebinding on drug dissociation in wash-out experiments.

Figure S2: Real-life examples of drug rebinding in wash-out experiments

Figure S3: Effect of dosing, $k_{\text{off}}$ and the rebinding factor on the target occupancy by different drugs.
References:


Robers MB, Dart ML, Woodroofe CC, Zimproch CH, Kirkland TA, Machliedt T et al. (2015). Target engagement and drug residence time can be observed in living cells with BRET. *Nat Commun* DOI: 10.1038/ncomms10091.


Supporting information

Figure S1: Simulated effect of rebinding on drug dissociation in wash-out experiments.
Figure S2: Real-life examples of drug rebinding in wash-out experiments.
Figure S3: Effect of dosing, $k_{\text{off}}$ and rebinding intensity on the target occupancy by different drugs.
Legends to figures:

A) One - compartment model

\[ \text{Inflow} \quad \begin{array}{c}
[L]_t + R \\
\xrightarrow{k_{on}} L.R \\
\xleftarrow{k_{off}} [L]_t \\
\end{array} \quad \begin{array}{c}
\text{elimination} \\
\xrightarrow{k_e} L \\
\end{array} \]

Two - compartment model

\[ \text{Plasma} \quad \begin{array}{c}
\text{Inflow} \\
\xrightarrow{k_a} \end{array} \quad \begin{array}{c}
[L]_t + R \\
\xrightarrow{k_{on}} [L_{on}]_t \\
\xleftarrow{k_{off}} [L]_t \\
\end{array} \quad \begin{array}{c}
\text{Effect compartment in tissue} \\
\xrightarrow{k_e} \end{array} \]

B) Classical equations

\[ \text{drug} \quad \begin{array}{c}
L + R \\
\xrightarrow{k_{on}} L.R \\
\xleftarrow{k_{off}} R \\
\end{array} \]

Equations for rebinding

\[ k_r = \frac{k_{on}}{1 + k_{on}[R]/k} \]

E.g. synaptic cleft

\[ L + R \xrightarrow{k_{on}} L.R \]

\[ k_r = \frac{k_{off}}{1 + k_{off}[R]/k} \]

Washout assay procedure

Simulated results

C) Drug dosing paradigms for simulations

- Constant \([L_{max}]\) paradigm
- Constant \([L_{max}]/K_D\) ratio paradigm

Iso-K_D situation
A) Living organisms are “open systems” wherein, after a single dosing, the concentration of free drug near the target first increases and then declines. In the simplest “one compartment” body model, the first-order rate constants $k_a$ and $k_e$ correspond to the inflow/input and the elimination/clearance of the drug respectively. Those govern how the concentration of free drug $[L]$ near the target, $R$, changes over time. Binding proceeds according to a reversible bimolecular mechanism; $k_{on}$ and $k_{off}$ are the association and the dissociation rate constants thereof. The more elaborate “two compartment” body model allows slow equilibration of the drug between the plasma compartment and a hypothetical target-bearing “effect compartment” within tissues (Holford and Scheiner, 1982; Gabrielsson et al., 2009). The rate by which the drug transfers between both compartments is governed by a single first-order constant, $k_{eo}$, for the present simulations (for the sake of simplicity). When the equilibration of free drug between both compartments is sufficient slow, its concentration in the effect compartment, $[L_e]$, will fluctuate at a slower pace than its concentration, $[L]$, in the plasma compartment.

B) Top left: inherent to the rate constants, $k_{on}$ and $k_{off}$, that govern a reversible mass-action type bimolecular binding mechanism, is the assumption that the drug and the target molecules are homogenously distributed in the solute and also that they are able to reach and leave one another by free three-dimensional (3D) diffusion.

Top right: in biological systems, targets like ion channels, receptors and enzymes are embedded in cell membranes and/or are present within the cell at high local density (Copeland, 2006). Cell walls and other obstacles that hinder free 3D diffusion of the drug (example shown for receptors that face a synaptic cleft) will promote drug rebinding; i.e. the ability of freshly dissociated drug molecules to experience several binding - unbinding sequences to the original target and/or to those nearby before drifting away (Perry et al., 1980; Goldstein et al., 1989; Goldstein and Dembo, 1995; Coombs and Goldstein; 2004; Gopalakrishnan et al., 2005). Research in different life sciences disciplines gave rise to same mathematical formulation of this mechanism: namely, $k_{on}$ is replaced by the ‘effective’ forward rate coefficient $k_f = k_{on}/(1 + k_{on}[R]/k)$ and $k_{off}$ is replaced by the ‘effective’ reverse rate coefficient $k_r = k_{off}/(1 + k_{on}[R]/k)$. These re-formulated equations do not affect the drug’s $K_D$. $k_f$ and $k_r$ are not constants since $[R]$ varies with the extent of target occupancy; the $k_{on}[R]/k$ product, which constitutes a metrics for rebinding, is maximal when the targets
are all free (i.e. when \([R] = [R_{\text{tot}}]\)) and decreases when the occupancy increases (see Figure 1 in the Supplementary Information section). The parameter, ‘k’, depends on the free drug’s diffusion rate and on the geometric characteristics of the target’s microenvironment (Coombs and Goldstein, 2004; Vauquelin and Charlton, 2010).

Bottom left: The ability of unlabelled competitive ligands/drugs to speed-up the dissociation of a pre-bound radioligand in a concentration-dependent fashion (Vauquelin and Van Liefde) represents the most commonly reported experimental manifestation of rebinding. To this end, targets are pre-incubated with radioligand and subsequently (preferably with an intermediary wash step to remove free radioligand) incubated in fresh wash-out medium alone (lane a) as well as in medium containing an excess of unlabelled competitive ligand (lane b) for different periods of time after which binding is measured (Vauquelin, 2012). A similar approach is theoretically also applicable when binding is measured by spectrophotometrically such as in BRET (Robers et al., 2015) and FRET-based assays.

Bottom right: simulated decline of radioligand binding when the wash-out is performed in the presence of an excess of unlabelled ligand (red curve, no rebinding - reflecting the genuine \(k_{\text{off}}\) of the radioligand) or in naïve wash-out medium (black curves account for increasing values of the rebinding factor, \(k_{\text{on}}[R_{\text{tot}}]/k\), a metric for the radioligand’s susceptibility to experience rebinding, see also Figure 1 in the Supplementary Information section). Dots refer to simulated data points.

C) Venn diagrams picturing the different in vivo dosing paradigms that were used to compare how the target occupancy by different drugs changes over time. Some of the earlier published simulations relied on a “constant \([L_{\text{max}}]\)“ paradigm such as in Figure 2D-F. The peak concentration of all the drugs (denoted as \([L_{\text{max}}]\) in this article) was the same whatever their binding rate constants and their \(K_D\). Other simulations relied on a, from the clinical perspective more representative, “constant \([L_{\text{max}}]/K_D\) ratio” paradigm such as in the ensuing figures of this article. The peak concentration of the drugs was adapted to permit the same maximal occupancy of the target by all the drugs (in the supposition that association and dissociation is too fast for binding kinetics to play any role). Both diagrams overlap when only drugs with the same \(K_D\) are compared to one another such as in Gabrielsson et al (2009).
Figure 2.
Simulated target occupancy vs. time plots by drugs with different binding kinetics upon wash-out of free drug (panel A), exponential decrease of the free drug concentration (Panels B and C) and after a single administration in vivo according to the constant \([L_{\text{max}}]\) paradigm (Panels E and F) and the constant \([L_{\text{max}}]/K_D\) ratio paradigm (Panels G to I). Simulations are based on solving the differential equations 1 to 7 in Table 1 as earlier described (Vauquelin et al., 2001) and occupancy curves are based on 50 - not presented - data points. Diss. \(t_{1/2}\) values (provided in the Panels) are in min and \(k_{\text{on}}\) values in \(M^{-1}\cdot\text{min}^{-1}\).
A) Simulated target occupancy, [RL], in “infinite dilution” wash-out conditions in where, after removal of free drug, L, pre-formed LR complexes are exposed to naïve medium for different time intervals (abscissa) till 24 h. [RL] is 90 % of [R_{tot}] at the start and decreases mono-exponentially. The rate thereof is independent of the initial target occupancy. Other parameters: k_{on} = 1.10^7 \text{M}^{-1}\text{min}^{-1}.

B and C) Same representation and drugs as in Panel A but [L] decreases mono-exponentially with t_{1/2} of 120 min. Black solid line stands for theoretical continuous equilibrium; the same color code as in Panel A applies to the drugs. At the start, [RL] amounts 50 or 90 % of [R_{tot}] and [L] and [RL] are at equilibrium. To facilitate comparison, [L] (dotted line) is normalized to reach the same apex as [RL].

D) Simulated [L] vs. time plots after \textit{in vivo} bolus administration. [L] Evolves with time during 24 h according to the Bateman function (Section 2.2) with k_{a} = 0.0115 \text{min}^{-1} (t_{1/2} = 1 h) for inflow and k_{e} = 0.00575 \text{min}^{-1} (t_{1/2} = 2 h) for elimination. \textit{f} (= 12.4 nM) was adjusted to yield [L_{max}] = 9 \times K_{D} (for 90 % occupancy at equilibrium) of a drug with k_{on} = 1.10^7 \text{M}^{-1}\text{min}^{-1} and k_{off} = 0.0069 \text{min}^{-1} (diss. t_{1/2} = 100 min).

E and F) Simulated [RL] vs. time plots after \textit{in vivo} bolus administration of different drugs according to the constant [L_{max}] paradigm (equations 3 and 4 in Table 1). The drug that was utilized for the curve in Panel D serves as “median” here (solid black line). & The other drugs have different k_{on} or k_{off} values; those values are normalized with respect to this median. In accordance with the present dosing paradigm, the [L] vs. time curve shown in Panel D (and thus also the value of f) applies to all the drugs here. Open circles in panel F refer to theoretical [RL_{max}]_{eq} values and when they are attained.

G and H) Simulated [RL] vs. time plots according to the constant [L_{max}]/K_{D} ratio paradigm (differential equations 5 and 6 in Table 1). All parameters are the same as for panel E (color code as insert in Panel E) except that \textit{f} is now adapted to yield [L_{max}] = 9 \times K_{D} (for 90 % occupancy at equilibrium) for all the drugs. Panel G: all curves overlap when changing k_{on} only. Panel H: effect of changing k_{off} only.

I) Alternative, [RL] vs [L] representation of the occupancy data shown in panel H. [L] reaches the same apex for all drugs when expressed in K_{D} -units.
Figure 3.
Simulated [RL] vs. time plots by drugs with different binding kinetics after in vivo dosing according to the constant $[L_{\text{max}}]/K_D$ ratio paradigm: effect of re-binding (differential equations 5 and 6 in Table 1). Free ligand parameters, $k_a$ and $k_e$ are the same as in Figure 2 and $[L_{\text{max}}] = 9 \times K_D$ for all drugs.
A to F) Effect of increasing the re-binding factor (color code in Panel A) for drugs with $k_{\text{on}} = 1.10^7 \text{M}^{-1}\text{min}^{-1}$ and, from one panel to the next, decreasing values of $k_{\text{off}}$ (given as diss $t_{1/2}$ in each panel).
G) Area under the curve (AUC) values for the 24 h post-administration period (in percent of continuous full occupancy) as a function of the dissociation $t_{1/2}$ of each drug (abscissa). Color code for the re-binding factor is given in Panel A.
H) Effect increasing $k_{\text{on}}$ from $1.10^6$ to $1.10^8 \text{M}^{-1}\text{min}^{-1}$ for drugs with constant $k_{\text{off}} = 0.0069 \text{min}^{-1}$ (diss. $t_{1/2} = 100 \text{ min}$) and re-binding factor = 10; all curves do overlap.
I to L) Open circles: effect of increasing $k_{\text{on}}$ (which also produces a proportional increase in the re-binding factor; starting from 1 at $k_{\text{on}} = 1.10^6 \text{M}^{-1}\text{min}^{-1}$) for drugs with constant diss. $t_{1/2} = 100 \text{ min}$. Solid lines: effect of increasing the diss. $t_{1/2}$ for drugs with constant $k_{\text{on}} = 1.10^7 \text{M}^{-1}\text{min}^{-1}$ and constant re-binding factor = 10. Log($k_{\text{on}}$) (for open circles) and diss $t_{1/2}$ values (for solid lines) are given in each panel. The curve for the “median” drug is shown in Panel H.
Figure 4.

Effect of two-compartment equilibration and rebinding and on the occupancy profile of a slow-dissociating drug.

A to C) Simulated [RL] vs. time plots by the same drug (k_{on} = 1.10^{7} M^{-1}.min^{-1} and dissociation t_{1/2} = 100 min, black line as control) and with, from left to right, increasing the rebinding factor (red line) and decreasing the equilibration k_{eo} (blue line, k_{eo} values are in min^{-1} and chosen to yield the same [RL\_max] as with rebinding). Evolution of [L] with time in the single (for rebinding) or plasma compartment (for 2-compartment model) is calculated by using the Bateman function for [L\_max] = 9 \times K_{D} and with the same values of k_{a} and k_{e}, as for Figure 2. Evolution of [L\_e] in the effect compartment with time is calculated by using equation 8 in Table 1.

D to F) Simulated [RL] vs. (plasma) concentration, [L], plots of the same drug and conditions as in Panels A to C (the same color code also applies).

G to I) Same [RL] vs. time plots with rebinding (red line) as in Panels A to C. To mimic ex-vivo experiments, simulations allow free drug to be removed (i.e. [L] is set to 0) either after 240 min or after 840 min (arrows) and the subsequent wash-out phase to be carried out in medium only (closed circles, [L] remains 0) or in medium containing a large excess of competing ligand to block rebinding (open circles, [L] remains 0 and the rebinding factor is also set to 0).
Effect of two-compartment equilibration (Panels A to C) and rebinding (Panels D to F) on the occupancy profile of a fast-dissociating drug. Drug binding parameters: $k_{\text{on}} = 1.10^7 \text{ M}^{-1}\text{.min}^{-1}$ and $k_{\text{off}} = 6.9 \text{ min}^{-1}$ (diss $t_{1/2} = 0.1\text{ min}$). $[L\text{max}] = 9 \times K_D$, $k_a$ and $k_e$ are the same as for Figure 2.

A and D) $[L]$ vs time plot (black line in both panels) in the plasma/single compartment and of the $[L_e]$ vs time plot in the effect compartment (colored lines in panel A) for the same $k_{\text{eo}}$ values as in Figure 4A-C.

B and C) $[RL]$ vs time plots (Panel B) and corresponding $[RL]$ vs $[L]$ plots (Panel C) for instantaneous equilibration between the plasma and effect compartments (control, black line) and equilibration with the $k_{\text{eo}}$ values given in Panel A (colored lines).

E and F) $[RL]$ vs time plots (Panel E) and corresponding $[RL]$ vs $[L]$ plots (Panel F) without rebinding (control, black line) or with the rebinding factors given in Panel D (colored lines are only shown in Panel F, where they are sufficiently separated from the control).
Figure 6.
Effect of rebinding and $k_{\text{off}}$ after repeated dosings. Simulated [RL] vs. time plots by drugs with different $k_{\text{off}}$ (vertical, corresponding to diss $t_{1/2}$ from 30 to 1000 min) according to the constant $[L_{\text{max}}]/K_D$ ratio paradigm: effect of rebinding (horizontal, rebinding factor 10 and 30) on repeated daily bolus administrations of the same dose (differential equations 5 and 6 in Table 1). $k_{\text{on}}$ (≈ $1.10^7$ M$^{-1}$min$^{-1}$) remains constant, free ligand parameters, $k_{\text{d}}$ and $k_{\text{e}}$, are the same as in Figure 2 and $[L_{\text{max}}] = 9 \times K_D$ for all drugs. After each dosing, $[L]$ is adapted to include the free drug that remains 24 h after the previous dosing. Color code for the consecutive dosings is given in Panel A. If the occupancy profile no longer changes after a given day, then the curves after that color-coded day are not shown.
Figure 7.
Quantitative aspects of the [RL] vs. time plots for the drugs shown in Figure 6 (in black, $[L_{max}] = 9 \times K_D$) and also of similar plots (not shown) for $[L_{max}] = 27 \times K_D$ (in red) and $90 \times K_D$ (in blue). $k_a$ and $k_e$, are the same as in Figure 2.

A to L) Area under the curves (in percent of continuous full occupancy) are presented as a function of the day of treatment (abscissa). Panels are presented in the same order as in Figure 6. Same color code (shown in panel A) in all panels.

M to O) Peak-to ensuing through ratios at day 8 are presented as a function of the drug’s dissociation $t_{1/2}$. Data correspond to $[L_{max}] = 9 \times, 27 \times$ and $90 \times K_D$. Data points also include Peak-to-through ratio’s for faster dissociating drugs. Same color code (shown in panel A) in all panels.
Legends to Tables:

Table 1:
Differential equations to follow the time (t)-dependent changes in target occupancy in a closed system and in an \textit{in vivo}-like and open system with dosing according to the constant $[L_{\text{max}}]$ and constant $[L_{\text{max}}]/K_D$ ratio paradigms. Part of equations at the right side account for rebinding. For equation 8, it is assumed that the equilibration of the drug between the two compartments does not affect $[L]_t$.

Table 1

<table>
<thead>
<tr>
<th>General:</th>
<th>for rebinding: append:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d[R]/dt = -d[RL]/dt$ with $[R_{\text{tot}}] = [R] + [RL]$ for all</td>
<td>Eq. 1</td>
</tr>
</tbody>
</table>

Bimolecular binding process with $[L]$ constant:

<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d[RL]/dt = (k_{\text{on}}.[R].[L] - k_{\text{off}}.[RL])/(1 + k_{\text{on}}.[R]/k)$</td>
<td>Eq. 2</td>
</tr>
</tbody>
</table>

\textit{In vivo} dosing according to constant $[L_{\text{max}}]$ paradigm, multiplying $k_{\text{on}}$ or $k_{\text{off}}$ by factor $X$:

| $X.k_{\text{on}}$: | $d[RL]/dt = (X.k_{\text{on}}).[L].[R] - k_{\text{off}}.[RL])/(1 + X.k_{\text{on}}).[R]/k)$ | Eq. 3 |
| $X.k_{\text{off}}$: | $d[RL]/dt = (k_{\text{on}}.[L].[R] - X.k_{\text{off}}).[RL])/(1 + k_{\text{on}}.[R]/k)$ | Eq. 4 |

\textit{In vivo} dosing according to constant $[C_{\text{max}}]/K_D$ paradigm

| $X.k_{\text{on}}$: | $d[RL]/dt = (X.k_{\text{on}}).[L].[R] - k_{\text{off}}.[RL])/(1 + X.k_{\text{on}}).[R]/k)$ | Eq. 5 |
| $X.k_{\text{off}}$: | $d[RL]/dt = (k_{\text{on}}.[X].[L].[R] - X.k_{\text{off}}).[RL])/(1 + k_{\text{on}}.[R]/k)$ | Eq. 6 |
| $X.k_{\text{off}}$ & $X.k_{\text{on}}$: | $d[RL]/dt = (X.k_{\text{on}}).[L].[R] - X.k_{\text{off}}).[RL])/(1 + X.k_{\text{on}}).[R]/k)$ | Eq. 7 |

Equilibration of the drug between the two compartments

$\frac{d[L_e]}{dt} = k_{eo}.[L]_t - k_{eo}.[L_e]$ | Eq. 8

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