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REVIEW

Long-lasting target binding and rebinding as mechanisms to prolong *in vivo* drug action

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An increasing number of examples in the literature suggest that the *in vivo* duration of drug action not only depends on macroscopic pharmacokinetic properties like plasma half-life and the time needed to equilibrate between the plasma and the effect compartments, but is also influenced by long-lasting target binding and rebinding. The present review combines information from different research areas and simulations to explore the nature of these mechanisms and the conditions in which they are most prevalent. Simulations reveal that these latter phenomena become especially influential when there is no longer sufficient free drug around to maintain high levels of receptor occupancy. There is not always a direct link between slow dissociation and long-lasting *in vivo* target protection, as the rate of free drug elimination from the effect compartment is also a key influencing factor. Local phenomena that hinder the diffusion of free drug molecules away from their target may allow them to consecutively bind to the same target and/or targets nearby (denoted as 'rebinding') even when their concentration in the bulk phase has already dropped to insignificant levels. The micro-anatomic properties of many effect compartments are likely to intensify this phenomenon. By mimicking the complexity of tissues, intact cells offer the opportunity to investigate both mechanisms under the same, physiologically relevant conditions.

Abbreviations

2D and 3D, two-dimensional and three-dimensional; BSA, bovine serum albumin; E_{max}, maximal effect; GPCR, G protein coupled receptor; K_D, equilibrium dissociation constant for bimolecular drug-target binding; k_f, effective forward rate coefficient; k_{off}, first-order dissociation rate constant of drug-target complex; k_{on}, second-order association rate constant of drug-target complex; k_r, effective reverse rate coefficient; PD, pharmacodynamics; PET, positron emission tomography; PK, pharmacokinetics; SPECT, Single photon emission computed tomography; τ, residence time of drug-target complex; $t_{1/2}$, half-life

Introduction

There are increasing examples in the literature illustrating that the long-lasting clinical action of drugs not only depends on their macroscopic pharmacokinetic properties like their plasma half-life and the time needed to equilibrate between the plasma and the effect compartments, but also on their ability to bring about long-lasting target binding. Less known but equally important are local phenomena that cause the drug molecules to accumulate near the target and/or hinder their free three-dimensional (3D) diffusion away from that target. Such hindrance may allow the same drug molecule to consecutively bind to the same target and/or targets nearby even when the free drug concentration further away has already dropped to insignificant levels. Information about the pharmacodynamic and local pharmacokinetic mechanisms that may contribute to long-lasting drug action is widely

dispersed in the literature. Indeed, these topics have not only caught attention of pharmacologists but also of, among others, biophysicians, physiologists, neurochemists and immunologists. The present review combines relevant information from these different research areas in an effort to have a better understanding of the nature of these mechanisms and the conditions in which they are most conspicuous.

Contribution of pharmacokinetics to long-lasting clinical efficacy

The efficacy and duration of drug action depends on both pharmacokinetic and pharmacodynamic factors. Pharmacokinetics (PK) is the discipline in pharmacology that investigates what the body does to the drug and, to this end, it focuses on the temporal evolution of a drug and its metabolites in serum as well as other body compartments like target and non-target tissues/organs. PK models mainly deal with the extent and rate of absorption, distribution (the dispersion of the drug and its potential metabolites between blood plasma and other body compartments), metabolism (the transformation of drug into active or inactive metabolites) and excretion. Pharmacodynamics (PD), on the other hand, examines what the drug does to the body (Holford and Sheiner, 1982). One of its aims is to study the time course of the drug's pharmacological effect after dosing (Derendorf *et al.*, 2000). Yet, the often observed time lag between the drug's response and its plasma concentration (as seen by a counterclockwise hysteresis loop of the corresponding plot, Figure 1) made it initially difficult to reconcile PK to PD. Accordingly, they were long considered as separate disciplines.

This came to an end by the introduction of a hypothetical compartment, the 'effect compartment', wherein the drug's target was defined to reside (Sheiner *et al.*, 1979; Holford and Sheiner, 1982). The time needed for the drug to equilibrate between the plasma and effect compartments could then be held responsible for the delayed response (Figure 1). This paved the way to a wide range of combined PK/PD models to link the temporal variations in both the drug concentration in different body compartments and its clinical effect (Meibohm and Derendorf, 1997; Csajka and Verotta, 2006). The hysteresis loop could be forced to collapse by linking the effect to the effect compartment concentration instead of the plasma concentration at least in some cases (Della Paschoa *et al.*, 1998), but not in all. Until a decade ago, the prevalent strategy for explaining the remaining hysteresis was to invoke a

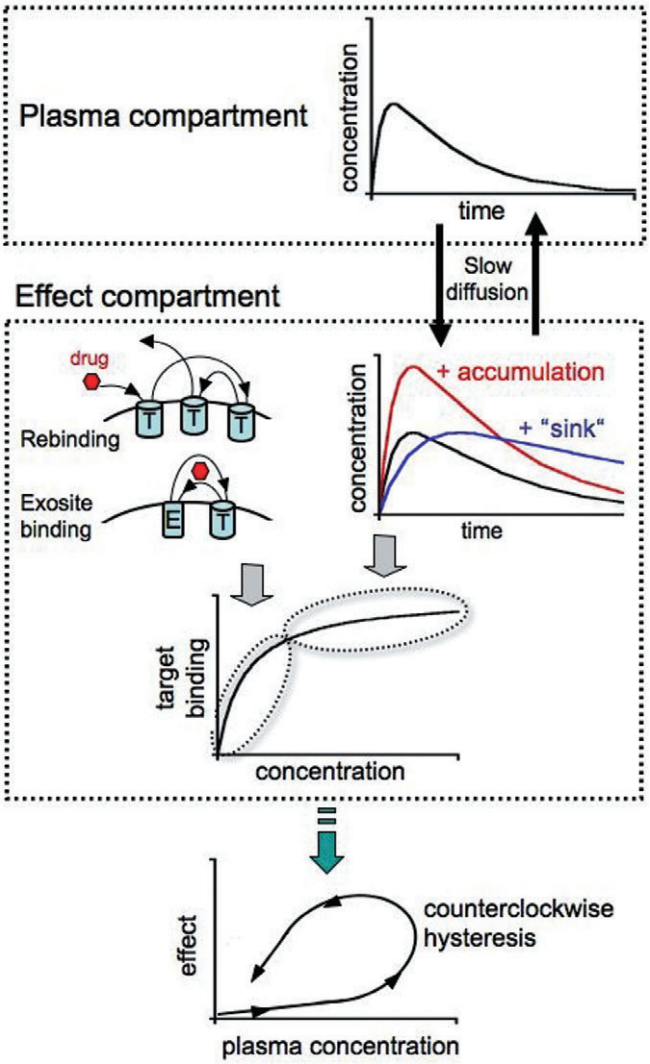

Figure 1

Factors contributing to long-lasting drug action. Classical pharmacokinetics (PK) contribution relates to a slow decline in free drug concentration in the plasma – and effect compartments (due to its slow metabolism and/or elimination) as well as to slow equilibration of the drug between both compartments. Local PK phenomena at the effect compartment that contribute to prolong high target occupancy include (i) increased drug concentration near the target by its uptake in the cell membrane from where it reaches the target (red curve); and (ii) prolonged presence of the drug near the target by its uptake in, and subsequent release from 'sinks' like cell membranes (i.e. the 'diffusion microkinetic' model, blue curve). When the target occupancy starts to decline, additional phenomena may contribute to prolong the occupancy. They include (i) slow dissociation of the drug-target complexes; (ii) ability of the drug to bind to multiple target (T) molecules before drifting away (i.e. rebinding); and (iii) ability of the drug to shuffle between the target and a (still hypothetical) high affinity 'exosite' (E). Combined, these phenomena may lead to a substantial delay between the drug's concentration in the plasma and the observed clinical effect (often presented by a counterclockwise hysteresis plot).

delay between the drug-target interaction and the generation of the response (Meibohm and Derendorf, 1997; Derendorf and Meibohm, 1999). Except for irreversibly acting drugs, those PK/PD models essentially regarded the amount of target-bound drug molecules to be in constant equilibrium with their free concentration at the effect site, so that attention could be focused on the potency/affinity and efficacy of the drug. It was thus sufficient to describe the drug-target interaction (and in most cases the thereby generated response) by equilibrium equations originating from the law of mass action and receptor theory (Hill, 1909; Ariens *et al.*, 1956; Black and Leff, 1983).

Newer, mechanism-based PK/PD models originated from a better understanding of the molecular mechanism of the drug-target interaction and the thereby triggered biochemical and physiological events. The investigation of such mechanisms is now also considered to be part of the PD domain (Derendorf *et al.*, 2000). Indeed, several *in vitro* studies have shed light on the hitherto overlooked importance of the rate of drug-target association and dissociation in linking the time-related changes in the concentration and the effect of a number of drugs (Shimada *et al.*, 1996; Äbelö *et al.*, 2001, 2006; Yassen *et al.*, 2005; Yun *et al.*, 2005). The importance of this kinetic concept is now gaining general recognition (Mager *et al.*, 2003; Ploeger *et al.*, 2009) and it has even led to the new 'residence time' terminology to describe the stability of the drug–target complexes (Copeland *et al.*, 2006; Tummino and Copeland, 2008; Zhang and Monsma, 2009). The residence time (τ) of the drug is inversely proportional to the first-order dissociation rate constant (k_{off}) of a binary drug-target complex. The term 'residence time' does not really epitomize a novel descriptive element in face of the more traditionally used dissociation half-life ($t_{1/2} = 0.69/k_{off}$) but, as it is intuitively more appealing, it is likely to gain ground.

Terminology and focus

Here, we will still use the traditional 'dissociation $t_{1/2}$' terminology and refer to 'receptor occupancy $t_{1/2}$' to describe the average time needed to liberate half of the initially occupied receptors under conditions in where drug association (i.e. in open systems, in where the free drug concentration is allowed to vary with time, Figure 2) or rebinding (i.e. when a drug can bind to several target sites before drifting away) can take place. Also, as targets may have diverse functionalities and locations, for simplicity we will mainly focus on ligand-membrane-associated receptor interactions in the ensuing sections.

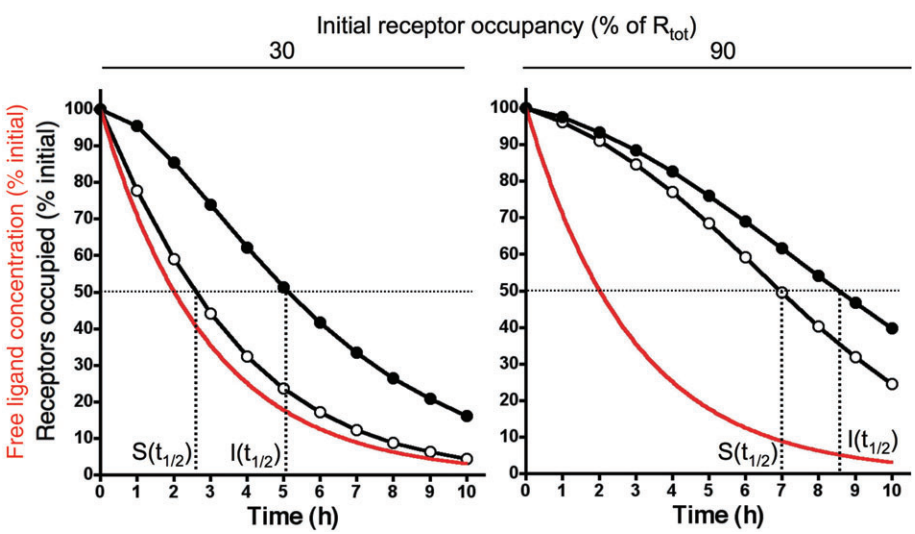

Figure 2

Receptor occupancy by a fast reversible (surmountable, S) ligand (○, k_{off} = 5.2 min^{-1}, k_{on} = 1 × 10^8 M^{-1} × min^{-1}) and a slow dissociating (insurmountable, I) ligand (●, k_{off} = 5.7 × 10^{-3} min^{-1}, k_{on} = 1 × 10^8 M^{-1} × min^{-1}) when the free ligand concentration decreases exponentially with time (with $t_{1/2}$ = 2 h, red curve). At the start, free and receptor bound ligands are at equilibrium and either 30% (left panel) or 90% (right panel) of the receptors (R_{tot}) are occupied. Data points were obtained by integrating the differential equations describing a first-order decrease in free ligand concentration and a bimolecular ligand-receptor interaction as previously described (Vauquelin *et al.*, 2001; Vauquelin and Van Liefde, 2006). Receptor occupancy half-life of the insurmountable ligand, $I(t_{1/2})$, is 2.0-fold the corresponding half-life by the surmountable ligand, $S(t_{1/2})$, at 30% initial occupancy. This value drops to 1.2-fold at 90% initial occupancy.

Contribution of drug-binding kinetics to clinical therapy

In drug-screening studies, ligand-receptor interactions are traditionally quantified in terms of affinity and efficacy only. Until recently, and besides a few exceptions (Leysen and Gommeren, 1986), only little attention has been paid to the kinetic properties of drug-target complexes. A likely reason for this is that the dissociation rate of the drug-target has historically been assumed to be too rapid to play a significant role. However, an increasing number of studies reveal that this is not always the case. Table 1 enumerates a few examples of slow-dissociating drugs, more can be found in Swinney (2004, 2006a) and Copeland *et al*. (2006). Among them, it was recently advanced that the slow dissociation of the muscarinic antagonist tiotropium bromide and of NK_1 Neurokinin receptor antagonists from their cognate receptors was the key to explain their prolonged duration of action (Disse *et al*., 1999; Lindström *et al*., 2007). These and other examples made it clear that the dissociation rates of the complexes may have far-reaching repercussions with respect to the drug discovery process. A number of interesting principles have also been advanced in the emanating review papers (Swinney, 2004, 2006a,b, 2008, 2009; Copeland *et al*., 2006; Vauquelin and Van Liefde, 2006; Tummino and Copeland, 2008; Van Liefde and Vauquelin, 2009; Zhang and Monsma, 2009). Among them, a distinction should be made about whether the drug-target complex is a source of adverse events or not. In the affirmative case, the complexes should be preferred to dissociate swiftly (i.e. to be readily surmountable) so that the occupancy of the target by its endogenous ligands is only dictated by their ability to compete with free drug molecules. This is the case for neuroleptics/antipsychotics used in clinical therapy to attenuate psychotic episodes (Creese *et al*., 1976). Neuroleptics of the first generation, the typical ones, displayed high potency and long-lasting D_2 dopamine receptor blockade. Yet, the incidental refractoriness of the striatal receptors in responding to fast fluctuations in the local dopamine concentration generated extrapyramidal side effects, including a Parkinsonian-like syndrome and tardive dyskinesia (Deutch, 1993). These adverse events are less prominent with second-generation, atypical neuroleptics because of their swifter dissociation from the D_2 dopamine receptors so that those in the striatum remain responsive to peaks in the dopamine concentration (Kapur and Seeman, 2000, 2001). Without intrinsic adverse events, also denoted as 'mechanism-based toxicity', long-lasting target-binding drugs are expected to be the most suitable agents for therapies that require continuing, high levels of target occupancy (Copeland *et al*., 2006; Swinney, 2006b; Zhang and Monsma, 2009). Compared with solely relying on the pharmacokinetic properties of the drug, such dissociation characteristics are likely to offer greater control over the issue of selectivity and toxicity. Indeed, slow dissociation is often associated with high binding affinity, and such drugs are likely to display pronounced target selectivity and a broad therapeutic window. Conversely, because of their lower affinity for collateral targets, such drugs are also likely to dissociate faster from undesirable, adverse event-mediating ones. In such case, the drug-primary target complexes will last longer than the undesirable complexes so that the beneficial effects of the drug are likely to outlast

Table 1

Example dissociation half-lifes of several G protein-coupled receptor ligands

Receptor	Ligand	Dissociation half-life ($t_{1/2}$)	References
M3 muscarinic receptor	Tiotropium	7.7 h	Dowling and Charlton, 2006
	Ipratropium	0.16 h	
CCR5 chemokine receptor	Maraviroc	136 h	Watson *et al*., 2005
	873140	>136 h	
μ opioid receptor	Alvimopan	30–44 min	Cassel *et al*., 2005
	N-methylnaloxone	0.46 min	
Histamine H1 receptor	Desloratidine	>8.7 h	Anthes *et al*., 2002
	GSK1004723	5.8 h	Slack *et al*., 2009
Angiotensin type 1 receptor	Candesartan	2 h	Vauquelin and Van Liefde, 2006
	Olmesartan	70 min	
Neurokinin 1 receptor	aprepitant	3.6 h	Hale *et al*., 1998

the adverse events. Yet, there are also examples where the dissociation rate and equilibrium affinity are not directly related (Leysen and Gommeren, 2004). Tiotropium exhibits similar binding affinity at both the M_3 muscrinic receptor, which provides efficacy, and the M_2 subtype that is responsible for cardiovascular side effects. However, it dissociates 10 times more rapidly from the M_2 receptor, displaying a beneficial profile that has been coined as 'kinetic selectivity' (Disse *et al.*, 1999). This clearly illustrates that it can be advantageous to not only rely on affinity measurements at equilibrium, but also to investigate the kinetic properties of new drugs (Dowling and Charlton, 2006). This may also be the case for allosteric ligands (which bind an alternative site to the endogenous agonist) that may, by their nature, have slow offset kinetics (Kenakin, 2008). Taken together, these considerations clearly illustrate the general concept that parameters that describe the *in vivo* selectivity of a drug are not necessarily static but could depend on the post-administration time.

Physiological relevance of drug dissociation rates

The stability of drug-target complexes varies a lot: from being irreversible in the case of covalently bound ligands such as β-haloalkylamines (Furchgott, 1966) and gastric proton pump inhibitors like omeprazole (Wallmark *et al.*, 1984) to very fast dissociating ones like atypical neuroleptics. Yet, most of the drugs will experience intermediate, more-or-less slow dissociation from their target (Swinney, 2004; Copeland *et al.*, 2006). Whether or not the dissociation rate of the drug has clinical relevance needs, first of all, to be regarded in light of the half-life of the free drug in the effect compartment. Moreover, in the case of receptor antagonists, attention should also be paid to the duration of receptor exposure to endogenous messengers like hormones and neurotransmitters (Kapur and Seeman, 2000, 2001; Vauquelin and Van Liefde, 2006).

This latter issue is clearly illustrated in classical organ bath experiments (and related intact cell experiments) in where the methodology consists of an initial exposure (for a reasonably long time period) of a tissue to a fixed concentration of the antagonist of interest, after which (still in the presence of the antagonist) the tissue is further incubated with increasing concentrations of an agonist (Leff and Martin, 1986). The response of the tissue is recorded at each agonist concentration and, compared with the control agonist concentration-response curve for a naïve tissue, the maximal response will obviously decline (in the absence of 'spare receptors') in case of an irreversibly binding antagonist. This type of antagonism is truly 'insurmountable' because the occupied receptors are forever refractive to agonist stimulation (Vauquelin *et al.*, 2002; Kenakin *et al.*, 2006). At the other extreme, antagonists may dissociate so swiftly that subsequently added agonist molecules can already access all the receptor molecules before the response is recorded. Such antagonists will only produce parallel rightward shifts of the agonist concentration-response curve. They are denoted as 'surmountable' because all the receptor molecules can get stimulated if the agonist concentration is high enough. In between resides a broad range of more-or-less slowly dissociating antagonists. In organ bath experiments, such antagonists will produce a mixed type (i.e. partially insurmountable) inhibition if the ensuing challenge with the agonist is to short to allow a new mass-action equilibrium to be fully operational before the response is measured (Lew *et al.*, 2000, 2001). The insurmountable behaviour of such antagonists only reflects a temporal hemi-equilibrium as it can be overcome by prolonging the agonist exposure. In practice, however, this may be impossible to achieve because of methodological constraints of the experiment. A final note of caution with respect to interpreting these experiments is that insurmountable antagonism could also result from allosteric/non-competitive interactions and that partial insurmountability could originate from the co-occurrence of two antagonist-bound receptor conformations/states with only one of them being surmountable (Vauquelin *et al.*, 2001; Swinney, 2004; Copeland *et al.*, 2006; Vauquelin and Van Liefde, 2006; Tummino and Copeland, 2008).

In contrast to intact and fractionated cell-based experiments and the usual organ bath experiments, administered drugs are gradually eliminated from a living organism. This implies that the concentration of free drug (or its active metabolite) in the effect compartment will also decline with time. If the drug in question dissociates swiftly from its receptor (i.e. when bound and free drug concentrations are in quasi-permanent equilibrium), the receptor occupancy $t_{1/2}$ will depend both on the rate of drug elimination from the effect compartment and the initial level of receptor occupancy (Vauquelin and Van Liefde, 2006; Szczuka *et al.*, 2009). Indeed, when the free drug concentration is at or below its equilibrium dissociation constant (K_D) for the receptor, the initial occupancy will only be modest and its decline will closely follow the decline in free drug concentration. On the other hand, when the free drug concentration is in large excess of its K_D, the

initial receptor occupancy will be nearly maximal and, because of the hyperbolic shape of E_{max} model-based saturation binding curve (Figure 1), it will decline much slower than the free drug concentration. Hence, high local drug concentrations in effect compartment will already lead to a long lasting effect by itself. However, being surmountable, such drugs will also continuously compete with other receptor ligands like metabolites and endogenous messengers for binding to the target.

On the other hand, the example of proton pump inhibitors (Wallmark *et al.*, 1984) clearly illustrates that the therapeutic action of irreversibly binding drugs might persist even after the free drug was fully eliminated from the effect compartment. In fact, their action can only be abolished by *de novo* synthesis of target molecules by the organism. Here again, the situation is more complex for the reversible but slow dissociating drugs. For such drugs, a first series of simulations already suggested that their binding could appreciably outlast that of a fast reversible/surmountable drug provided that their dissociation is slower than their elimination from the relevant compartment (Vauquelin and Van Liefde, 2006; Tummino and Copeland, 2008). The present simulations constitute an extension thereof by focusing on the potential impact of the free drug elimination $t_{1/2}$ (from 15 min to 12 h) and of the initial fraction of receptor occupancy (30, 80 and 95%) on the receptor occupancy $t_{1/2}$. Three drugs were compared, a surmountable/fast reversible one, a slow dissociating one (dissociation $t_{1/2}$ = 2 h) and a quasi-irreversible one (dissociation $t_{1/2}$ = 12 h). An example of the results is illustrated in Figure 2 and an example of derived receptor occupancy $t_{1/2}$ versus free drug elimination $t_{1/2}$ plots is illustrated in Figure 3 for initial 80% receptor occupancy. An interesting observation is that the latter plots are quasi-linear and parallel for the three drugs examined and that their intercept with the ordinate closely amounts their dissociation $t_{1/2}$-values (hence, the vertical separation between the plots corresponds to the difference in their dissociation $t_{1/2}$).

We here arbitrarily opted for a ≥50% increase in receptor occupancy $t_{1/2}$ to establish a threshold above which the binding of an insurmountable drug appreciably outlast that of a fast reversible one. In Figure 3, this rule applies to the sections of the plots that are located above the red line. Upon inspection, these and additional (not shown) simulations suggest that it is not the elimination rate itself but rather the ratio between the dissociation and elimination rates that determines whether the binding of an insurmountable drug will appreciably outlast that of the fast reversible one. Moreover, the threshold is not constant as it depends on the initial

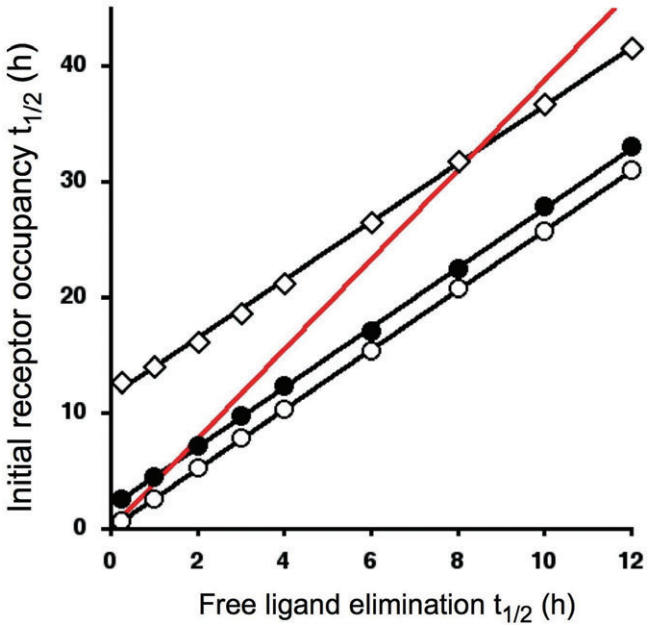

Figure 3

Receptor occupancy $t_{1/2}$ as a function of the free ligand's elimination $t_{1/2}$. Experimental set-up and data generation and analysis are the same as in Figure 2. Initial receptor occupancy amounts 80% of R_{tot}. Three ligands were compared: a fast reversible (surmountable, S) one (○, k_{off} = 5.2 min^{-1}), a slow dissociating one (●, k_{off} = 5.7 × 10^{-3} min^{-1}) and a quasi-irreversible one (◇, k_{off} = 4.8 × 10^{-4} min^{-1}) and k_{on} = 1 × 10^{8} M^{-1} × min^{-1} for all. Binding of the latter ligands was arbitrarily considered to outlast that of the surmountable one when their receptor occupancy $t_{1/2}$ exceed 1.5 times S($t_{1/2}$) (this rule applies to the sections of the plots that are located above the red line representing this threshold value). Table 2 lists such threshold free ligand elimination $t_{1/2}$-values for the different fractions of initial receptor occupancy.

Table 2

Threshold $t_{1/2}$-values (in h) for insurmountable ligand elimination below which their receptor occupancy $t_{1/2}$ outlasts that of the surmountable ligand by ≥50%: influence of the initial fraction of occupied receptors and the ligand's dissociation $t_{1/2}$

Ligand dissociation $t_{1/2}$ (h)	Initial receptor occupancy (% of R_{tot})		
	30	80	95
◇, 2	4	1.5	0.7
●, 12	26	8.3	4.5

degree of receptor occupancy (Table 2). At 30% initial occupancy, it is already sufficient for the dissociation to take place at half the elimination rate; at 80%, the dissociation already needs to be a little slower; while at 95%, the dissociation already needs to be about three times slower. The reason for this dependence may be found in the hyperbolic shape

of a saturation binding curve at equilibrium and the resulting impact of the extent of initial receptor occupancy upon the rate by which it declines in case of a surmountable agonist, as discussed previously (Figure 1). Taken together, the present, extended simulations confirm that the terms 'slow dissociation' and 'long-lasting receptor blockade' by antagonists should not be used in strict synonymy.

Nevertheless, even when the elimination of a free antagonist drug is rate limiting, slow dissociation/insurmountablility may still improve its efficacy *in vivo*, particularly in systems with rapid and transient signalling characteristics (Vauquelin and Van Liefde, 2006). The most extreme example of such a system is neurotransmission, where neurotransmitter is released locally within a synapse at very high concentrations, but which is very rapidly removed by reuptake and metabolic routes. In the synapse, the concentration of agonist is so high that if left to reach equilibrium, it would compete off the antagonist and bind to all the receptors. The extremely transient nature of high neurotransmitter concentration, however, means that most antagonist ligands will have a long-enough duration at the receptor to effectively antagonize the agonist over the very short period of high agonist concentrations, completely blocking transmission even in situations of very high agonist concentrations.

Related situations may also occur in other systems, for example, chemotaxis of leukocytes into areas of inflammation. Ligands that competitively antagonize chemotactic receptors equilibrate with the receptor in the periphery and only come into contact with high concentrations of chemoattractant for brief periods as they pass through an inflammatory focus. The concentrations of chemoattractant can be very high in those foci, up to 1000-fold their EC_{50} for the CXCR2 chemokine receptors on human neutrophils (Nocker *et al.*, 1996; Traves *et al.*, 2002). A rapidly dissociating compound will re-equilibrate with the chemoattractant in the time it takes for the leukocyte to pass the area of inflammation, possibly resulting in an incomplete blockade of infiltration into tissue. In these situations, receptor blockade will depend upon the relative concentrations of each ligand. A slowly dissociating ligand will, however, remain largely bound to the receptor during the time taken to pass the inflammatory focus, effectively antagonizing the chemoattractant, thereby demonstrating higher efficacy. Recently, the kinetic characteristics of Sch527123, a novel CXCR2 antagonist developed by Schering Plough, have been directly determined using a tritiated version of the molecule. The binding of [^{3}H]Sch527123 to CXCR2 receptors in CHO cells was found to be slowly reversible with a dissociation $t_{1/2}$ of approximately 22 h (Gonsiorek *et al.*, 2007). It is possible that this slow dissociation contributes to its highly efficacious inhibition of pulmonary inflammation in animal models (Chapman *et al.*, 2007).

Evidence for rebinding in radioligand dissociation experiments

Several experimental strategies can be deployed to determine the dissociation rate of drug-receptor complexes (Table 3) (Vauquelin and Szczuka, 2007). When a drug is available in a radiolabelled form and if it displays sufficiently high affinity and specificity for its cognate receptor, its dissociation can be directly measured by following the time-course of its specific/receptor binding under wash-out conditions. All these experiments require a preliminary incubation of the receptor-containing preparation with the radioligand but, for the subsequent wash-out, four major experimental paradigms can be discerned depending on whether or not the radioligand-containing medium is replaced by fresh medium and whether or not an excess of unlabelled competitive ligand is supplied (Table 3). Exposure to

Table 3

Methods for measuring radioligand dissociation rates: advantages and limitations

Method	Removal of free radioligand after pre-incubation with receptor	Excess unlabelled ligand in wash- out medium	Advantages and limitations
1	Yes	Yes	Correct k_{off}*
2	No	Yes	Correct k_{off}*
3	Yes	No	Rebinding possible
4	Only dilution	No	Rebinding possible and approximate k_{off}#

*Only if both radioligand and unlabelled ligand interact competitively, that is, when they bind to overlapping sites at the receptor.
#Due to the establishment of a new mass-action equilibrium with diluted free radioligand in the wash-out medium.

Table 4

Selected situations in where rebinding is likely to prolong drug/ligand-target binding

Ligand	Target	System
(1) Thyrotropin	Thyrotropin receptors	Human thyroid membranes
(2) Naloxone	μ opioid receptor	Recombinant CHO cells
(3) Sartans	AT_1 angiotensin II receptors	Recombinant CHO cells
(4) Spipeone	D_2 dopamine receptors	Recombinant CHO cells
(5) Rimonabant, taranabant	CB_1 cannabinoid receptors	Recombinant CHO cells
(6) Epidermal growth factor	EGF receptors	A431 cells
(7) Dinitrophenyl ligands	Cell-surface IgE	Rat basophilic leukemia cells
(8) Fibroblast growth factor-2	Heparan sulphate proteoglycans	Vascular smooth muscle cells
(9) Diprenorphine, naloxone	Opioid receptors	Rat brain, *in vivo*
(10) CH 23390	D_1 dopamine receptors	Rat brain, *in vivo*
(11) Fasciculin 2	Acetylcholinesterase	Neuromuscular junction
(12) Lectins	Selectin	Cell-cell interactions
(13) Peptide-MHC class II ligands	T cell receptors	Cell-cell interactions
(14) Antibodies (also monomeric)	Immobilized ampicillin	BIAcore biosensor

Many other experimental observations in where target binding is shortened by the presence of an excess of other drugs/ligands have been published but they were, rightfully or not, merely commented in view of non-competitive interactions. References: (1) Powell-Jones *et al.*, 1979; (2) Spivak *et al.*, 2006; (3) Fierens *et al.*, 1999; Le *et al.*, 2007; (4) Packeu *et al.*, 2008 (5) Szczuka *et al.*, 2009; Wennerberg *et al.*, 2010; (6) Wiley (1988); (7) Goldstein *et al.*, 1989; Posner *et al.*, 1992; (8) Chu *et al.*, 2004; (9) Perry *et al.*, 1980; (10) Gifford *et al.*, 1998; (11) Krejci *et al.*, 2006; (12) Lou *et al.*, 2006; Thomas, 2006; (13) Germain, 1997; (14) Nieba *et al.*, 1996.

new medium containing an excess of unlabelled ligand (Method 1) ensures its fast occupancy of all the free receptor sites so that the binding of any remaining free radioligand or even receptor-dissociated radioligand molecules is effectively prevented (Limbird, 1996). This is presumably the safest method determining the radioligand's genuine dissociation rate. Even without replacing the medium, the mere addition of unlabelled ligand (Method 2) will reduce radioligand association. Yet, for this to be effective, one needs to ensure that the concentration of the unlabelled ligand is high enough. One way to deal with this issue is dilute the medium before adding the unlabelled ligand. With this in mind, it is clearly less satisfactory for radioligand dissociation to be monitored in fresh medium alone (i.e. without unlabelled ligand, Method 3) and even worse if the initial incubation medium is not replaced by, but simply diluted with an excess of fresh medium (Method 4).

Because dissociated radioligand molecules are capable of binding again to their cognate receptor molecules with Method 3 but not with Method 1, a comparison of the time-wise decline of the specific radioligand binding under both wash-out conditions offers an elegant approach to find out whether such 'rebinding' or 'reassociation' can effectively take place. By applying this approach on intact plated recombinant cell lines expressing the human G protein coupled receptors of interest, we have already detected such rebinding behaviour for a number of radiolabelled antagonist molecules. They include the AT_1-type angiotensin II receptor antagonists [^{3}H]-candesartan, [^{3}H]-olmesartan and [^{3}H]-telmisartan; the D_2 dopamine receptor antagonists [^{3}H]-spiperone and [^{3}H]-raclopride (but to a much lower extent); and the CB_1 cannabinoid receptor antagonists [^{3}H]-rimonabant and [^{3}H]-taranabant (Fierens *et al.*, 1999; Le *et al.*, 2007; Packeu *et al.*, 2008; Szczuka *et al.*, 2009; Wennerberg *et al.*, 2010). Compared with the mono-exponential decline in radioligand binding and the involvement of the total receptor population when the wash-out takes place in an excess of unlabelled ligand, dissociation seems to be greatly delayed if not halted entirely. In this case, dissociation only appears to occur from a fraction of the labelled receptor molecules when the wash-out takes place in fresh medium only. Upon surveying the literature, it appears that such differences in dissociation behaviour have also been observed for other receptor systems (Table 4). Yet, rather than evoking the rebinding hypothesis, authors tend to focus on the faster dissociation of the radioligand in the presence of the unlabelled ligand (Vauquelin and Szczuka, 2007). Indeed, this phenomenon is generally regarded as a hallmark for negative cooperativity between the radioligand and the unlabelled ligand

and, in turn, could point to the occurrence of allosteric interactions (Park *et al.*, 1990; Limbird, 1996; Gill *et al.*, 1999; Urizar *et al.*, 2005). To be more precise, by binding to a remote/'allosteric' site, the unlabelled ligand is able to trigger/favour a conformational change of the receptor molecule (or multimeric complex therof) and this could enhance the dissociation of the radioligand from the receptor's 'orthosteric' site (Christopoulos and Kenakin, 2003).

Fortunately, at least two criteria offer the opportunity to discriminate between the 'rebinding' and the 'allosteric interaction' models. First is to compare the dissociation behaviour of the radioligand when the wash-out is carried out in the presence of increasing concentrations of different unlabelled ligands of the examined receptor. If those unlabelled ligands produce a concentration-dependent increase in the radioligand's (apparent) dissociation rate with the same order of potencies as in competition binding experiments and if their maximal effect is the same, it is already very likely that the 'rebinding' model accounts for the observations. Indeed, it should already be quite unlikely for distinct allosterically acting ligands to trigger/favour the same conformational change of the receptor and, hence, to increase the dissociation rate of the orthostetic ligand (i.e. the radioligand) to the same extent (Christopoulos and Kenakin, 2003). Second, the likelihood of this interpretation is further supported if the unlabelled equivalent of the radioligand increases the radioligand's dissociation rate to the same extent as the other unlabelled ligands and if the radioligand binding experiments show no evidence of negative cooperativity (i.e. when the Hill coefficient of the saturation binding plot is close to 1). The radiolabelled antagonists [^{3}H]-candesartan and [^{3}H]-spiperone have already been inspected according to those criteria and, at least for those there is ample evidence for rebinding to take place if the wash-out takes place in fresh medium only (i.e. Method 3) (Fierens *et al.*, 1999; Packeu *et al.*, 2008).

Finally, although the most widely used procedure to prevent radioligand rebinding is to include an excess of unlabelled ligand in the wash-out medium, Goldstein *et al.* (1989) proposed that the same result can also be obtained by capturing free radioligand molecules with high affinity binding proteins such as specific antibodies. As bovine serum albumin (BSA) also avidly binds certain classes of drugs (Simard *et al.*, 2006); it could also act as an external 'sink' in such cases. In agreement, rebinding of [^{3}H]-candesartan and [^{3}H]-taranabant to their receptors was indeed partly prevented by addition of 1% w/v BSA in the wash-out medium (Fierens *et al.*, 1999; Szczuka *et al.*, 2009). The effect of these soluble proteins also points at rebinding as it can only take place if the radioligand is free in solution (and, hence, physically separated from its receptor) for some time. An alternative interpretation of such phenomena in terms of an unfavourable conformational change of the receptor seems less probable because it requires the soluble protein to interact with an allosteric site at the receptor.

Functional organ bath 'wash-out' experiments are often also carried out in compliance with Method 3. In such experiments, pre-incubation of the target tissue with the drug is followed by brief wash steps and a final, long-term incubation of the tissue in the wash-out medium. If the drug is an agonist, the remaining response can be recorded at distinct time intervals (Austin *et al.*, 2003). If the drug is an antagonist, the tissue needs to be challenged with and excess of agonist at distinct time intervals and the response monitored soon thereafter. By this method, the time-wise loss of receptor occupancy by the antagonist can be indirectly deduced from the restoration in receptor's responsiveness. Using this strategy, and in agreement with the [^{3}H]-candesartan dissociation experiments in medium only, there was only a very slow recovery of the contractile response of candesartan-pretreated rabbit aortic strips and rat portal veins to angiotensin II (Ojima *et al.*, 1997; Morsing *et al.*, 1999). Similar functional wash-out experiments, but this time with AT_1 receptor expressing recombinant Chinese Hamster Ovary cells, also yielded a very slow recovery of the response (here measured in terms of inositol phosphates accumulation) (Vanderheyden *et al.*, 1999). Together with the radioligand dissociation experiments, these functional wash-out experiments are indicative for the physiological relevance of rebinding phenomena.

Current mechanistic interpretations of rebinding

According to a widespread pharmacologist's view, radioligand molecules that are freshly dissociated from their receptor distribute rapidly all over the wash-out medium so that its rebinding is only a matter of restoring a new mass-action equilibrium between free and bound radioligand (Sutter *et al.*, 1979; Limbird, 1996). This implies that rebinding should only be preeminent when the radioligand (or antagonist in functional wash-out experiments) displays very high affinity for its receptor. Although [^{3}H]-candesartan complied with this requisite, [^{3}H]-telmisartan also experienced considerable rebinding under the same experimental conditions and this despite its much lower affinity for the AT_1

receptor (Fierens *et al.*, 1999; Le *et al.*, 2007). Moreover, computer-assisted simulations revealed that, for simply restoring a new mass-action equilibrium between homogenously distributed free and bound [^{3}H]candesartan, the free concentration had to be eight times higher than the total amount of radioligand that what was left in the wells (Fierens *et al.*, 1999). As alternative explanation, it was suggested that the released radioligand molecules are subjected to local accumulation in the vicinity of their receptors. This interpretation joins the already ancient 'unstirred layer' concept (Silhavy *et al.*, 1975). Although earlier dismissed in the context of ligand-membrane associated receptor interactions (Limbird and Lefkowitz, 1976; De Meyts *et al.*, 1977), this concept has recently been restored in honour (Copeland *et al.*, 2006; Spivak *et al.*, 2006).

Brownian motion refers to the random walks of molecules in solution resulting from their continuous collisions with molecules of the solvent and a large set of such random walks can be formulated by the classical equations of macroscopic diffusion. In this respect, bimolecular ligand-receptor interactions are customarily described by equations that apply to solutes, that is, molecules that are homogeneously distributed in a solvent with unrestricted 3D diffusion therein. Classical PK models also assume that compartments are homogenous and well stirred (Gifford *et al.*, 1998; Pang *et al.*, 2007). Even in this situation, diffusion already plays a role in the ligand's association and dissociation characteristics. Indeed, according to the prevalent biophysical model (DeLisi, 1981; DeLisi and Wiegel, 1981), binding between the ligand (L) and its receptor (R) proceeds according to a two-step process (Figure 4). Diffusion of both molecules will first bring them sufficiently close together to form a so-called 'encounter complex' [(L. . . R)] and it is only then that the reversible binding process (formation of L.R) can take place. This model separates the reversible ligand-receptor binding in two components: one depends on the transport characteristics of both partners (designated by the diffusive rate constants k_+ and k.), while the other one depends on the interaction mechanism (designated by the reaction rate constants k_1 and k_{-1}). It can also be represented by a single-step process with the same mathematical form as the classical one describing such interaction between soluble molecules in a well-mixed system except that the 'fundamental' association and dissociation rate constants (k_{on} and k_{off}) are replaced by the 'effective' forward rate coefficient k_f and the 'effective' reverse rate coefficient k_r (Figure 4) (Goldstein and Dembo, 1995). However, k_f and k_r are not constant in other circumstances (please see next) and this is why the term 'coefficient' is to be preferred.

Yet, although essentially overlooked in pharmacology, receptors have only a very restricted diffusion capability when they are confined to cell membranes and even the very presence of the membrane itself already constitutes a hindrance/wall for free 3D diffusion of the ligand. Because of these topological characteristics of the receptor environment, calculations according to biophysical models indicate that ligands are more prone to experience rebinding phenomena than in a situation where the same receptors are uniformly distributed in solution (Berg and Purcell, 1977; DeLisi, 1981; Shoup and Szabo, 1982; Goldstein *et al.*, 1989; Goldstein and Dembo, 1995). Indeed, based on the premises that the membrane constitutes a reflective surface and that the receptors only constitute a minor portion of this surface, most ligand molecules should first hit the membrane at a non-receptor locus. Calculations by Berg and Purcell (1977) according to this model further revealed that, after that initial collision, the ligands are most likely to make several more encounters with non-receptor loci at the membrane before they interact with an associated receptor molecule or drift away. Because of this initial tendency of the ligand to remain in close proximity of the membrane surface, it has a higher probability to hit a receptor within a given time-span as compared with the situation where ligand and receptor molecules are homogeneously dispersed in solution. The same reasoning is obviously also applicable to ligand molecules that freshly dissociated form their cognate receptor and it was calculated that this could already substantially increase the probability of rebinding when the cell surface carried 3500 receptor molecules or more (DeLisi, 1981). In this respect, it is important to realize that the initial binding to and release of a ligand from the receptor (as well as its adsorption into and release from the plasma membrane, see further) is in most cases not a necessary condition for rebinding to take place. Indeed, compared with simply bouncing off the cell wall, binding to a receptor only delays the ligand but it does not affect the subsequent spatial trajectory of the dissociated ligand (Andrews, 2005). It is only if a bi/multidentate ligand can carry out caterpillar-like crawling between adjacent receptors/targets that this initial interaction may count (Levin *et al.*, 2002; Gopalakrishnan *et al.*, 2005).

Based on these and other biophysical considerations, Goldstein and colleagues could equate k_f and k_r as a function of the 'fundamental' rate constants k_{on} and k_{off} for membrane-associated receptor molecules in different spatial contexts, including a

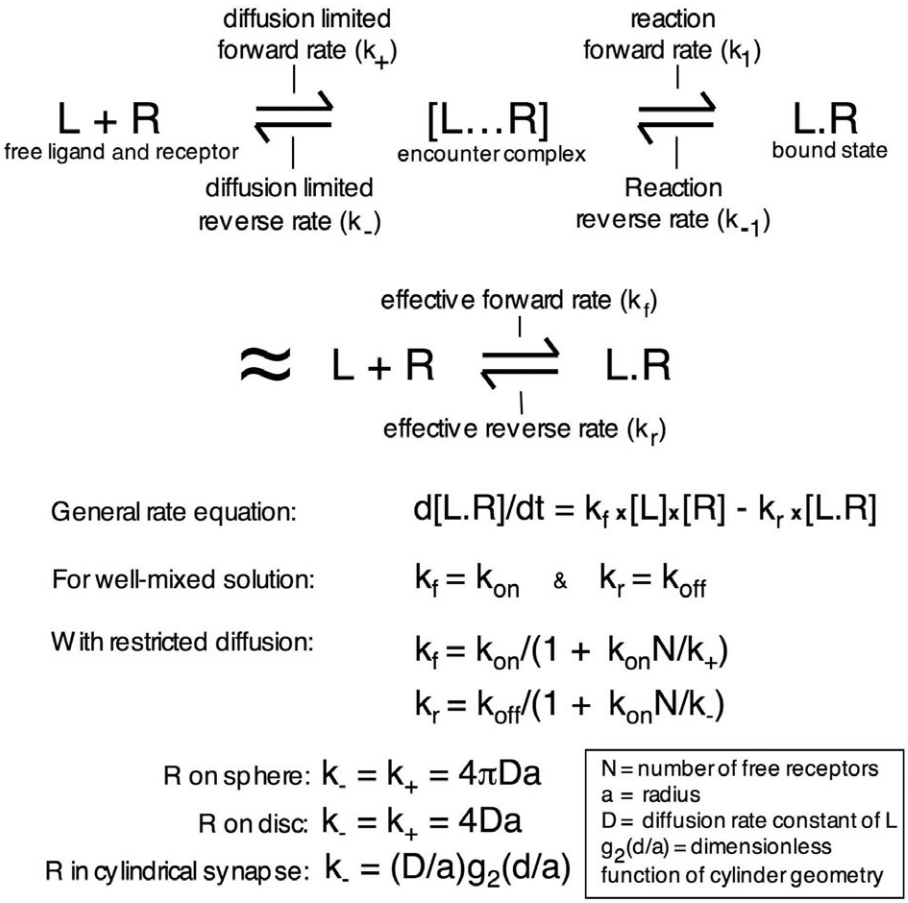

Figure 4

Schematic representation of drug (L) – receptor (R) binding with the intermediate formation of an encounter complex [L. . . R] (DeLisi, 1981; DeLisi and Wiegel, 1981). This binding will also be represented by a single-step process with the 'effective' forward rate cefficient $k_f = k_+k_1/(k_- + k_1)$ and the 'effective' reverse rate coefficient $k_r = k_-k_{-1}/(k_- + k_1)$. In a well-mixed system, these 'effective' rate coefficients correspond to the 'fundamental' association and dissociation rate constants (k_{on} and k_{off}). As k_f and k_r are not always constant, such as in the following situations, the term 'coefficient' is to be preferred. When receptors are present at the cell surface, k_f and k_r are related to k_{on} and k_{off} (respectively) by a factor that depends on k_{on}, the diffusion rate of the ligand (D), the geometry of the system and the amount of free receptors available (N) (Goldstein *et al.*, 1989; Goldstein and Dembo, 1995; Coombs and Goldstein, 2004).

whole surface of a spherical cell, a cluster-mimicking disc or the bottom of a synapse-mimicking cylinder (Goldstein and Wiegel, 1983; Goldstein *et al.*, 1989; Posner *et al.*, 1992; Goldstein and Dembo, 1995; Coombs and Goldstein, 2004). In these equations, k_f and k_r are still proportional to k_{on} and k_{off} respectively (Figure 4). An important property of the proportionality factor (F) is that it is not constant but that it depends on the amount of free receptor molecules (N) at the surface in question. This allows F to account for rebinding phenomena when radioligand dissociation is measured in fresh medium only. Indeed, it is only at full initial receptor occupancy (i.e. when $n = 0$) that F is equal to one (Figure 4). Then, as more receptors become free with time, F (and, hence, k_r) starts to decrease as well. As illustrated by the simulations in Figure 5, this may give rise to (at first sight) only partial radioligand dissociation and lead to interpretations in terms of receptor heterogeneity. Moreover, F depends on k_{on} but not on K_D. This distinction is important as it implies that a ligand's aptness to undergo rebinding will depend on its reaction forward rate constant rather than on its affinity for the receptor. Finally, F also depends on the diffusive reverse rate constant (k_-, characterizing the flux of ligands away from the receptor-bearing surface), which not only depends on the translational diffusion coefficient (D) of the ligand (receptor diffusion is to slow to be accounted for) but also on geometric characteristics of the receptor environment (Figure 4). In synapses or similar flat cavities, rebinding is promoted by the propensity of dissociated ligand molecules to return to the receptor-bearing surface many times before they manage to diffuse out of the cavity (Coombs and Goldstein, 2004).

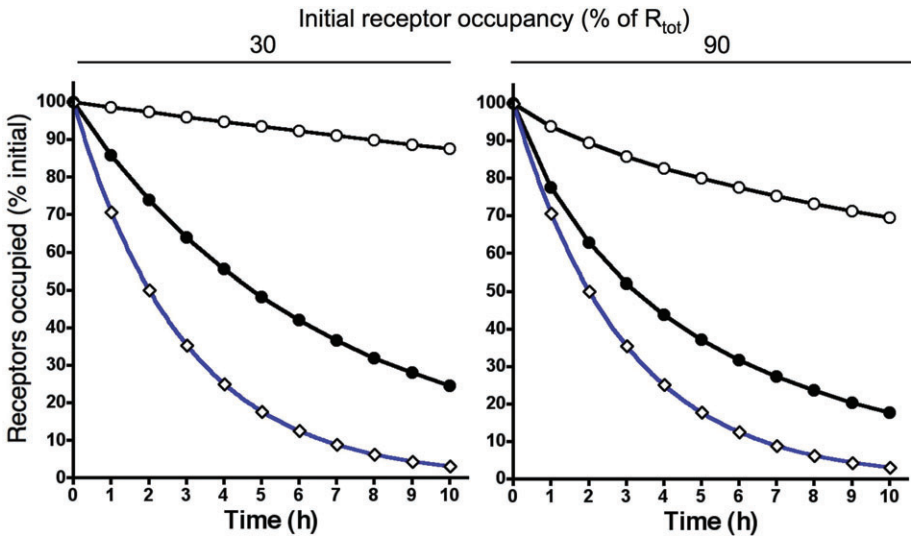

Figure 5

Effect of rebinding on the time-wise decline in receptor occupancy by a slow dissociating ligand ($k_{off} = 5.7 \times 10^{-3}$ min^{-1}, $k_{on} = 1 \times 10^{8}$ $M^{-1} \times min^{-1}$) after wash-out of the free ligand. At the start, free and receptor bound ligand is at equilibrium and either 30% (left panel) or 90% (right panel) of the receptors (R_{tot}) are occupied. Data points were obtained by integrating the differential equations describing a bimolecular ligand-receptor interaction without rebinding (◇ and curve in blue, 'effective' reverse rate coefficient $k_r = k_{off}$) or with rebinding ($k_r = k_{off}/(1 + k_{on}.[R].k_-)$ at low total surface receptor density (●, 3.89×10^{12} receptors·cm^{-2} and $k_{on}.[R].k_- = 1.22$ and 0.174 at 30 and 90% initial occupancy, respectively) and 20-fold higher surface receptor density (○, $k_{on}.[R].k_- = 24.3$ and 3.47 at 30 and 90% initial occupancy respectively). Note that because of the greater availability of free receptors for ligand rebinding, this process is more outspoken at high total receptor density and low initial receptor occupancy.

Restricted ligand diffusion plays an important role in biology and it is therefore not surprising that alternative mathematical descriptions have also been proposed to model reactions that take place under dimensional constraints such as those at the surface of membranes, in unstirred media and when a large fraction of the volume is taken up by macromolecules (i.e. 'macromolecular crowding') (Pang *et al.*, 2007). Among them, the 'fractal reaction kinetics' model (Kopelman, 1988) only necessitates a simple adaptation of the classical formalism to describe reversible bimolecular reactions based on unrestricted 3D reactant diffusion. In this model, the association rate is not constant throughout the reaction course but it decreases in an exponential fashion with time: that is, $k_{(t)} = k_o.t^{-h}$, where $k_{(t)}$ and k_o is the rate coefficient at time t and t = 0, respectively, and h is a measure of the 'dimensionality' of the system. This time-wise decrease in k reflects the tendency of the reactants to undergo spatial segregation when a reaction progresses in an unstirred medium with limited diffusion. Although the use of this equation has been well documented in case of association phenomena (Kopelman, 1988; Schnell and Turner, 2004), it is also likely to apply to rebinding phenomena. Indeed, despite the different form and the heuristic nature of the equation, the 'fractal reaction kinetics' approach can depict rebinding phenomena in closely the same way as the approach devised by Goldstein and coworkers (please see legend of Figure 5)

Taken together, ligand rebinding implies that a fraction of the receptor-dissociated ligand molecules choose to bind again rather than to drift away. This may result in consecutive binding of the same ligand molecule to neighbouring receptor molecules in the cell membrane. The said interpretation of the rebinding phenomenon differs from the still prevailing 'novel mass action equilibrium' model (Limbird, 1996) that it is a highly localized phenomenon and, hence, does not require the presence of ligand in the aqueous bulk phase.

Physiological relevance of, and traits that promote ligand rebinding

One of the most evocative studies supporting the *in vivo* relevance of ligand rebinding was presented by Gifford *et al.* (1998). Following a bolus injection of the D_1 dopamine receptor antagonist [^{3}H]-SCH 23390 in rats, the tracer accumulated far more in the striatum as in the cerebellum (the control region with very low D_1 dopamine receptor content) and this striatal content was found to decline very slowly with time despite the fast breakdown of the tracer in plasma. Similarly, when the rats were sacrificed soon after the injection, a very slow decrease

in the radioligand content of the 'still living' striatal slices was noticed in wash-out medium alone. This slow decrease was attributed to rebinding because it could be dramatically enhanced by the presence of an excess of unlabelled ligand. Related experiments, but in where brain was homogenized instead of sliced, failed to show accelerated radioligand release in the presence of unlabelled ligand (Sadée *et al.*, 1982). This was imputed to the disruption of a diffusion boundary next to the receptor sites (receptor micro-compartment) during the homogenization process. Ligand rebinding has also been invoked by many more authors to explain unusually long *in vivo* binding properties of radioligands as well as positron emission tomography and single photon emission computed tomography radiotracers, but usually without providing further experimenal evidence (e.g. Perry *et al.*, 1980; Frost and Wagner, 1984; Votaw *et al.*, 1993; Frost, 2001).

Drug rebinding is likely to be favoured by the anatomical and physiological complexity of higher organisms. Indeed, biophysical models suggest that this phenomenon is favoured by a high local receptor density, a high reaction forward rate constant and obstacles that hinder the flux of the ligands from the receptor-bearing surface.

Of particular note is that the cell plasma membrane is not a homogenous structure but contains ordered cholesterol-rich microdomains such as lipid rafts with an average diameter in the 100–200 nm range and caveolae that manifest themselves as 50–100 nm 'flask shaped' invaginations and also contain cholesterol-binding protein caveolin-1 (Pike, 2003). Many receptors seem to be concentrated in such microdomains along with coupling factors, effector enzymes and substrates. Because of the spatial proximity of the interacting components, signal transduction would occur rapidly and efficiently. β_2-adrenoceptors in cardiomyocytes constitute a typical example thereof (Okamoto *et al.*, 1998; Ostrom *et al.*, 2001; Steinberg, 2004). Because it is more likely for a dissociated drug to bind to a nearby receptor/target than to one that is far away, these and other clustering types have been proposed to promote rebinding phenomena (Andrews, 2005).

Rebinding phenomena may also take place in optical biosensors, such as the BIAcore, in where surface-immobilized target molecules are subjected to a flow of soluble target-binding analytes (Nieba *et al.*, 1996). To minimize this effect, low levels of immobilized target as well as high flow rates have been recommended (Karlsson and Falt, 1997). In an elegant study with cell monolayers, Spivak *et al.* (2006) also noticed that the equilibration between free ligand molecules and their receptors deep within the clefts separating the cells is delayed when compared with the situation at the top surface and that this delay could be minimized by increasing the flow rate. In this respect, it should be noted that *in vivo*, most receptors are not expected to be subject to any convective stirring of significance. Indeed, they are likely to face rather flat liquid-filled cavities such as neuronal synapses, neuroendocrine and neuromuscular junctions and other interstitial spaces. Moreover, even free circulating immune cells are able to form immunological synapses among themselves as well as with endothelial cells (Taub *et al.*, 1993; Grakoui *et al.*, 1999). In complex tissues, ligand diffusion in and out of such extracellular spaces is also delayed by their need to traverse tortuous paths with eventually blind pockets within the tissue and the fractal networks of the microcirculatory system (Lovich *et al.*, 2001; Dokoumetzidis *et al.*, 2004; Hrabctová and Nicholson, 2004), so that rebinding may take place unabated. Additionally, the extracellular matrix (forming a structural net composed of different types of macromolecules) is also likely to constitute an obstacle to the escape of dissociated ligands by free 3D diffusion (Dityatev and Schacher, 2006; Vargová and Syková, 2008). This latter phenomenon is also referred to 'macromolecular crowding' and, in the same vein, the cytoskeleton and high protein content (occupying up to 40% of the cell volume) also constitute diffusion obstacles inside the cell (Hall and Minton, 2003; Rivas *et al.*, 2004; Schnell and Turner, 2004). In this respect, free ligand diffusion in extracellular spaces may also be affected by a number of pathological conditions (Vargová and Syková, 2008). For example, ischaemia/anoxia has been observed to go along with cell swelling in the central nervous system (Syková *et al.*, 1999; Homola *et al.*, 2006). This swelling causes a substantial reduction in the extracellular space volume and a concomitant increase in the concentration of existing free ligand diffusion barriers therein. Such conditions could also favour ligand rebinding.

Finally, small ligand molecules and peptides with adequate physicochemical properties are able to undergo electrostatic interactions with head groups of membrane phospholipids and reside close to the membrane- solution interface (Kane *et al.*, 2008). If hydrophobic interactions with lipid hydrocarbon chains prevail, ligands may even become completely embedded within the lipid bilayer (Herbette *et al.*, 1988; Sargent *et al.*, 1988). Such ligands are able to undergo 2D diffusion at the cell surface and, because of this 'reduction of dimensionality', they are expected to find their receptor targets with greater ease than exclusively via 3D diffusion in solution (Adam and Delbrück, 1968). Moreover, while a molecule may never cross its starting point

again if diffusing in dimension D > 2, it is likely to stay in the vicinity of the starting point when the diffusion is restricted to a lower dimension (de Gennes, 1982; Kopelman, 1988; Berry, 2002). Taken together, this restricted diffusion is already likely to favour ligand rebinding phenomena in its own right. Moreover, a number of such ligands have been proposed to gain access to their binding site at their receptor via lateral diffusion between the receptor's membrane-spanning α-helical domains, that is, without having to leave the membrane (reviewed in Vauquelin and Packeu, 2009). Here, two additional mechanisms could act hand in hand with the 'reduction of dimensionality' phenomenon to exacerbate the rebinding of such ligands. First, ligand–lipid interactions could exert translational, conformational and orientational constraints on such ligands (Sargent and Schwyzer, 1986; Rhodes *et al.*, 1992; Schwyzer, 1995; Bader *et al.*, 2001). While they have no unique 3D structure in solution, they could acquire well-defined conformations in the membrane that are favourable for receptor binding (Sargent *et al.*, 1988; Contreras *et al.*, 2001). Second, membrane-spanning proteins will also create macromolecular obstacles to the unrestricted 2D-diffusion of such ligands (Berry, 2002). Taken together, rebinding could become very pronounced in the case of membrane-incorporated ligands when the above-mentioned phenomena act together to reduce their diffusive reverse rate constant, k_-, and to increase their reaction forward rate constant, k_1, for binding to the receptor target.

Rebinding and other local pharmacokinetic mechanisms that prolong drug action

Importantly, by partitioning in the membrane, ligand molecules could acquire a high local concentration therein (Vauquelin and Packeu, 2009). Such ligands could also be released slowly back in the aqueous phase (Figure 1). This combination of properties was proposed to explain the comparatively gentle onset and long-lasting clinical action of the lipophilic dihydropyridine lacidipine despite its short plasma half-life (Herbette, 1994). It also constitutes the core of the 'diffusion microkinetic' model that was advanced to explain the long-lasting bronchodilatory effect of β_2-adrenoceptor agonists like formoterol and salmeterol. This model stipulates that the plasma membrane can act as a depot/reservoir for the ligand rather than merely functioning as an inert substratum for the receptor (Anderson, 1993). Yet, the diffusion microkinetic model alone failed to explain why, in organ bath experiments, the long-lasting airway smooth muscle dilatory effect of salmeterol was independent of its initial concentration and the magnitude of its effect at the onset of the wash-out (Johnson *et al.*, 1993). To overcome this limitation, it was proposed that this agonist is able to undergo specific and long-lasting binding to auxiliary sites, 'exosites', located either at the receptors themselves or in their immediate vicinity in the membrane (Johnson *et al.*, 1993; Johnson and Coleman, 1995; Coleman *et al.*, 1996). This theory was originally proposed by Rocha e Silva (1969) to explain the persistent antagonistic activity at histamine H_1 receptors and, more recently, also for the muscarinic acetylcholine receptor agonist xanomeline (Christopoulos *et al.*, 1998). The presence of an exosite implies that, once dissociated from the active site of a receptor, the ligand is not free to diffuse away (Figure 1). As such, the exosite and rebinding models are rather convergent. Indeed, the rebinding model could be regarded to represent a variant of the exosite model if one assumes that clustered receptors are exosites for one another (Szczuka *et al.*, 2009). Alternatively, exosite binding could be regarded to represent an extreme form of rebinding as it calls a halt to the flux of free ligand molecules away from the receptor rather than merely reducing that flux.

If other pharmacokinetic mechanisms fail, the diffusion microkinetic model is certainly sufficient to prolong drug action as long as the plasma membrane (or any other depot close to the receptors) harbours a sufficiently high level of drug molecules. It is only when this mechanism exhausts (i.e. at low drug levels) that additional local pharmacokinetic phenomena like rebinding and/or exosite binding may start to play a preeminent role (Figure 1). To date, no solid experimental evidence has been presented in support of the participation of any of these latter mechanisms in the case of drugs like salmeterol and this may explain why this topic is still a matter of debate (Coleman, 2009; Szczuka *et al.*, 2009). However, to settle this issue, there seems to be a consensus about the need for dedicated studies on simple experimental systems before moving to more complex ones like intact tissues.

Concluding remarks

Whereas pharmacokinetic modelling has traditionally been based on *in vivo* studies, newer mechanism-based PK/PD models also rely on *in vitro* observations dealing with drug transport, metabolism, distribution and target binding (Derendorf and Meibohm, 1999; Pang *et al.*, 2007; Ploeger *et al.*, 2009). In this respect, *in vitro* information about

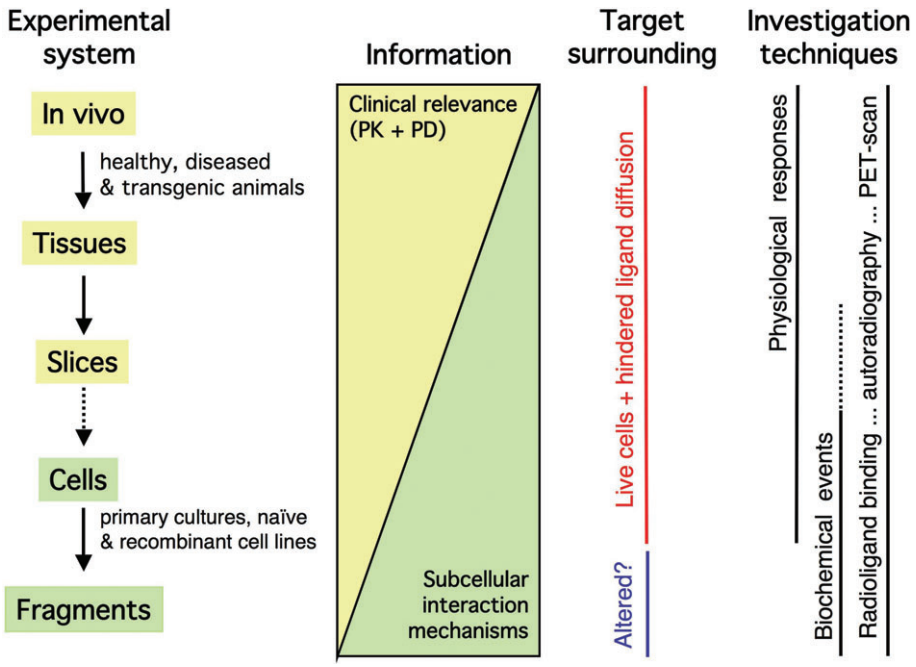

Figure 6

Pharmacological models and approaches: relevance of the obtained information. Nowadays, ligand-receptor/target interactions can be studied with a large variety of pre-clinical experimental systems (from cell fragments such as isolated membranes to *in vivo* animal models) and techniques (including the direct receptor labeling by radioligands and the measurement of receptor-mediated physiological responses and, by biochemical techniques, the intermediary intracellular signalling cascades). Of note is that the physiological/clinical relevance of the obtained information increases with the complexity of the experimental system. On the other hand, the observations on simple experimental systems are not only faster and cheaper but also easier to interpret in terms of molecular mechanisms. Yet, it is important to be aware that membrane-associated receptors/targets lose part of their natural environment when a cell is disrupted. This may alter their ligand interaction properties. PD, pharmacodynamics; PET, positron emission tomography; PK, pharmacokinetics.

drug-target binding kinetics is hitherto most often based on observations with intact tissues (such as organ bath wash-out experiments) and/or on radioligand binding experiments on isolated cell membrane preparations (Figure 6). Despite the unquestionable physiological relevance of the former approach, intact tissues may be too complex to provide straightforward information about drug-target interaction kinetics as well as pharmacokinetic mechanisms that take place at the sub-cellular/molecular scale (Coleman, 2009; Szczuka *et al.*, 2009). At first sight, experiments on membrane preparations seem to be more appropriate were it not that the so-obtained ligand-receptor dissociation rates sometimes markedly deviate from the rates observed in intact cells (Hara *et al.*, 1998; Fierens *et al.*, 2002; Vauquelin and Packeu, 2009). Little attention has been paid to this issue yet, but many more examples could be discovered if further dedicated comparative studies were to be performed. The rationale for this distinct behaviour is still unclear but a potential hint towards an explanation is that leaky cells already behave membrane-like (Verheijen *et al.*, 2004; Vauquelin and Packeu, 2009). This highlights the fact that membrane receptors (and membrane-associated proteins in general) lose part of their natural environment when a cell is disrupted. This natural environment entails differences between the ionic composition and the redox potential at both sides of the plasma membrane as well as the organizing role of the cytoskeleton. Additionally, receptor molecules that are normally hidden from the cell surface due to their presence in intracellular compartments also become accessible when the cells get disrupted. Intact plated cells seem to offer a compromise between these two *in vitro* experimental systems (Figure 6). From the physiological point of view, they certainly offer a more relevant environment than the membrane preparations, not only because they are alive but also because, to some extent, they mimic the microanatomic complexity of intact tissues (Spivak *et al.*, 2006; Grießner *et al.*, 2009). Yet, compared with those tissues, they offer far greater experimental flexibility, including the ability to compare radioligand binding and functional data under the same experimental conditions, easy wash-out and the ability to parallel determinations on

equivalent samples in each well rather than consecutive determinations on each tissue (Vauquelin *et al.*, 2002). Recombinant cell systems offer additional advantages, including a better focus on the receptors of interest and the use of the naïve parent cells for the detection of receptor-unrelated phenomena. Therefore, they offer a good compromise between the information that can be obtained at the pharmacodynamic level (which requires a simple system) and the local pharmacokinetic level (which requires a tissue-like microanatomical complexity).

Taken together, long-lasting clinical action of drugs not only depends on their macroscopic pharmacokinetic properties but also on their ability to bring about long-lasting occupancy of their target. Whereas slow dissociation starts to acquire general recognition, rebinding offers an additional but still little investigated option for enhancing the duration of drug action. By mimicking the complexity of tissues, intact cells offer the opportunity to investigate both mechanisms under the same, physiologically relevant conditions.

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Conflict of interest

None.

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