OPINION

Drug–target residence time and its implications for lead optimization

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Abstract | Much of drug discovery today is predicated on the concept of selective targeting of particular bioactive macromolecules by low-molecular-mass drugs. The binding of drugs to their macromolecular targets is therefore seen as paramount for pharmacological activity. *In vitro* assessment of drug–target interactions is classically quantified in terms of binding parameters such as IC_{50} or K_d. This article presents an alternative perspective on drug optimization in terms of drug–target binary complex residence time, as quantified by the dissociative half-life of the drug–target binary complex. We describe the potential advantages of long residence time in terms of duration of pharmacological effect and target selectivity.

Around the turn of the twentieth century Paul Ehrlich coined a phrase that quickly became the *sine qua non* of modern pharmacology and molecular medicine: *corpora non agunt nisi fixate* ("a substance will not work unless it is bound")[1]. Or, translated into more contemporary parlance, a drug is efficacious only so long as it is bound to, and modulating the action of, its physiological target(s). There are, of course, exceptions to this generalization, such that modulation of a target's function can persist subsequent to drug dissociation (as a result, for example, of hysteretic conformational effects and post-translational modifications, leading to desensitization, tachyphylaxis, oligomerization and other effects)[2,3]. Nevertheless, as captured in Ehrlich's aphorism, there is today a clear understanding that the formation of a binary complex between a drug molecule and its macromolecular target is of primary importance as the progenitor of *in vivo* drug action. As a result, much of early-phase drug discovery is focused on the optimization of target affinity and selectivity for drug candidates. Target affinity is typically assessed in cell-free assays that measure compound binding to a target directly (for example, receptor-binding assays) or indirectly by the effect of a compound on the biochemical activity of the target (such as enzyme activity assays). Typically, these *in vitro* measurements are performed under closed-system conditions (see Glossary Box), in which the target is exposed to an invariant concentration of the compound throughout the period of the measurement. Accordingly, these assay conditions only poorly mimic the temporal concentrations and duration of exposure that the compound exerts against its target in an *in vivo* setting. In such a closed system, the extent of compound binding to a target is commonly quantified in terms of the half-maximal inhibitory concentration (IC_{50}) or, more correctly, by an equilibrium dissociation constant (K_d) for the drug–target binary complex (referred to here as the receptor–ligand complex, regardless of the nature of the macromolecular target (receptor, enzyme, ion channel and so on) or the ligand).

The equilibrium dissociation constant, measured *in vitro*, can sometimes be directly related to the *in vivo* efficacy of a ligand. More often, however, one observes a qualitative, rather than quantitative, relationship between these measures, especially when the duration of the pharmacological effect is an important component of overall *in vivo* efficacy. In fact, Swinney[4] has recently provided a perspective on the value of drug candidates that behave in ways that are distinct from the classical view of rapid equilibrium binding of drugs to their targets. He suggests that departure from such a rapid equilibrium interaction, in which the final receptor–ligand equilibrium generally reflects a more potent interaction and is only established after a lengthy incubation period, comprises an important determinant of the *in vivo* effectiveness of small-molecule drugs.

In this current perspective we propose that one of the most crucial factors for sustained drug efficacy *in vivo* is not the apparent affinity of the drug for its target *per se*, but rather the residence time of the drug molecule on its molecular target. The term 'residence time' has several unrelated meanings in the field of pharmacology. For the purposes of this article, we define the term binary complex residence time (referred to here simply as 'residence time') as the period for which the receptor is occupied by a ligand.

As stated above, at the molecular level drug action can only occur (in most cases) when the drug is physically bound to its molecular target. The longer the receptor–ligand complex endures, the longer the drug's effect, either *in vitro* or *in vivo*. This is not a new concept, but rather an underlying — yet often unspoken (and under-appreciated) — assumption of both the equilibrium and non-equilibrium models of drug action. For *in vitro* experiments, in which the receptor is continuously exposed to an invariant concentration of ligand, the residence time of the receptor–ligand complex is related to, and appropriately quantified by, the final equilibrium dissociation constant[4,5]. By contrast, in the open system of *in vivo* experiments, the concentration of ligand to which a target receptor is exposed is no longer constant, but varies with time after dosing and is often influenced by factors other than the molecular determinants of receptor–ligand interaction. Accordingly, we propose that for *in vivo* situations the duration of efficacy of a ligand is no longer well described by the *in vitro* measured dissociation constant, but rather depends on the rate of receptor–ligand association (that is, the association rate constant or on-rate (k_{on})) and, most critically, on the dissociation rate constant, or off-rate (k_{off}), of the receptor–ligand complex. The off-rate can be simply translated into a dissociative half-life for the receptor–ligand complex, and this half-life is a direct measure of the residence time.

We propose that *in vitro* measurement of the dissociative half-life is a crucial metric of compound optimization, and could be a key indicator of *in vivo* duration of efficacy. We therefore recommend that *in vitro* assessment of compound interactions with targets should include measurements of both the final equilibrium dissociation constant and the dissociative half-life (BOX 1). Moreover, we suggest that differences in the dissociative half-life of a drug from its intended (primary) target and those of other cellular proteins to which it might also bind (secondary targets) could provide an informative measure of drug selectivity as it relates to the extent of potential adverse effects *in vivo*.

Box 1 | **Experimental methods for measuring dissociative half-life**

There are four major categories of experimental methods that are commonly used to follow the kinetics of receptor–ligand complex dissociation. These are described briefly below.

Separation methods[33–35]. These methods take advantage of a convenient detection label on the ligand, such as a spectroscopic signal or radioisotope incorporation. The receptor and ligand are combined and allowed to incubate until equilibrium is established. The free ligand is then rapidly separated from the receptor-bound ligand population by filtration or chromatographic means. Alternatively, separation can be achieved by binding the receptor to a solid support by the use of an affinity-based tag, such as an immobilized antibody to the receptor, biotinylation of the receptor and binding to immobilized avidin, and related methods.

Spectroscopic differentiation[33,34]. Here the researcher takes advantage of a unique spectroscopic signal that is associated with the bound or free state of one of the binding partners. A common example of this is the technique of fluorescence polarization (FP) spectroscopy, in which the polarization of fluorescence is related to the tumbling rate, and therefore molecular size, of the fluorescent species. A fluorescent ligand in solution tumbles rapidly and so has a characteristically low FP value. When that ligand is bound to a macromolecular receptor its tumbling rate is now retarded, and so its value of FP is dramatically increased. A second example of such methods is the use of intrinsic protein fluorescence from the receptor. The fluorescent amino acids of the receptor (tryptophan and sometimes tyrosine) are often exquisitely sensitive to changes in the conformation of the receptor brought about by ligand binding. As such, the fluorescence wavelength maximum and signal amplitude can be used to monitor binding events. After establishing characteristic signal values for the free and bound states, the dissociative half-life is determined as follows. The free components are combined at high concentrations and allowed to reach equilibrium. The equilibrium is then perturbed by a large and rapid dilution of the components into buffer. The signal is then monitored as a function of time as dissociation occurs.

Recovery of biological activity[5,36]. Methods in this category are usually used in conjunction with inhibitors of enzyme targets, when a convenient assay of enzymatic activity is available to the researcher. As above, one combines the free enzyme and inhibitor at high concentrations until equilibrium is established. The sample is then rapidly diluted into the enzyme assay system to perturb the equilibrium between inhibitors and enzyme, and to initiate the enzymatic reaction with substrate molecules. As the enzyme dissociates from inhibitor it becomes available to react with substrate and to contribute to the assay signal. The degree of enzymatic activity is monitored over time to define the rate of inhibitor dissociation, and from these data the value of the dissociative half-life can be calculated.

Immobilized binding partner methods[37,38]. Here the ligand or receptor is immobilized to a surface and the binding partner is free to associate and/or dissociate from the immobilized species. A variety of immunological and other detection methods fall into this category. Today, the most commonly used methodology in this category is measurement of refractive index (RI) changes due to the formation of a binary complex, using instruments exemplified by the BIAcore instrument. Buffer is flowed over the surface containing the immobilized species to establish a baseline RI value. This solution is then replaced by one containing the binding partner. As the solution flows over the surface, binding ensues with an accompanying change in RI value until equilibrium is established. The rate of association and the final equilibrium concentration of the binary complex can thereby be determined. Once equilibrium is reached, the flow solution can once again be replaced by the original buffer to perturb the equilibrium. The rate of return of the RI value to the pre-binding level is followed to define the dissociation rate.

Theoretical background

The overall duration of a receptor–ligand complex will be influenced by both the rate of ligand association (k_{on}) and the rate of binary complex dissociation (k_{off})[5], as discussed below. However, the residence time is, by our definition, related only to the rate of complex dissociation. A macroscopic analogy is one's residence time at a hotel, an industry term used to calculate the charges for a hotel stay. This is not influenced by how long it takes one to arrive at the hotel (due to traffic or other delays), but the period of time between check in (analogous to the bound state) and check out (analogous to dissociation). In many scientific instances the concept of residence time is quantified by the time constant, also called the relaxation constant (τ), which is the reciprocal of the dissociation rate constant ($1/k_{off}$)[5]. In pharmacology and drug discovery, the term half-life is much more familiar. We can define the dissociative half-life of a receptor–ligand complex as the relaxation time multiplied by 0.693 ($-\ln[0.5]$) and thereby relate this half-life (half-life = $0.693/k_{off}$) directly to the residence time[5].

Two common situations are well known with respect to receptor–ligand equilibria in pharmacology. The first is a simple one-step association and one-step dissociation process (see equation 1).

$$R \underset{k_2}{\overset{k_1[L]}{\rightleftharpoons}} RL \qquad (1)$$

In this situation k_{on} and k_{off} are identical to the microscopic rate constants k_1 and k_2. The second situation involves receptor isomerization after initial ligand binding, leading to a much higher-affinity final complex (RL*; see equation 2).

$$R \underset{k_2}{\overset{k_1[L]}{\rightleftharpoons}} RL \underset{k_4}{\overset{k_3}{\rightleftharpoons}} RL^* \qquad (2)$$

In this case, the term k_{off} is composed of microscopic rate constants associated with both forward and reverse steps in receptor isomerization, as well as the rate constant for dissociation of the initial RL complex back to the free ligand and free receptor states ($k_{off} = k_2k_4/(k_2 + k_3 + k_4)$). This additional complexity can significantly extend the overall dissociative half-life in the commonly encountered situation of slow receptor isomerization.

In either of these cases, the rate of ligand association with the receptor is determined both by the value of k_1 (that is, k_{on}) and by the concentration of ligand. Although the rate of ligand association can have an important impact on pharmacology and, as described later, on the duration of the receptor–ligand complex, there are several constraints on this rate that minimize its utility as a metric for preclinical compound optimization. First, although values of k_{on} vary considerably among receptor–ligand pairs[6], this rate constant is ultimately limited by the rate of diffusion of the two binding partners in physiological solutions (typically, this places an upper limit on the value of k_{on} of ~10^8–10^9 M^{-1} s^{-1}). Although in favourable cases medicinal chemistry efforts could theoretically be focused on accelerating the rate at which slow-binding ligands reach this limit, there is no mechanism available to the medicinal chemist to overcome the diffusion limit. Second, the physico-chemical steps involved in receptor–ligand association, such as diffusion, desolvation and molecular orbital orientation, are difficult for medicinal chemists to affect in any systematic way. Attempts to vary these steps through structure–activity relationship (SAR) studies are therefore purely empirical in nature. Third, the rate

of association depends not only on the value of k_{on} but also on the concentration of ligand. As such, a smaller value of k_{on} can often be compensated for by merely increasing the ligand concentration (that is, the dose). Finally, it must be recognized that in the open system of *in vivo* pharmacology, multiple steps such as absorption, distribution and clearance affect the local concentration of ligand that arrives at the receptor-harbouring tissue and therefore the rate of encounter between a dosed ligand and a tissue-associated receptor. Often, these *in vivo* steps confer a significant limitation on the overall rate of biomolecular encounters, such that the microscopic rate constant k_{on} is no longer the main determinant of complex formation.

By contrast, the dissociation rate is independent of these pharmacological factors, and of the local concentration of ligand. Rather, k_{off} is entirely dependent on specific interactions (such as changes in protein conformation, nonpolar forces, hydrogen bonds, van der Waals interactions and so on) between the ligand and its target binding pocket. Optimization of such interactions, and consequently of the dissociation rate, is therefore the fundamental value of medicinal chemistry in terms of the iterative optimization of receptor–ligand affinity, as assessed through SAR studies.

In fact it is often the case that SAR studies that are guided by IC_{50} or K_d measurements in the closed systems of *in vitro* assays principally lead to the optimization of dissociative half-life, although this is usually unrecognized by the experimenters. One might argue, then, that the need for optimization of dissociative half-lives is already fulfilled by standard *in vitro* measurements, and that there is no need for the more complete assessment of both K_d and k_{off} values. Two points are worth noting in this regard. First, optimization of K_d and k_{off} values are not always convergent within the SAR of a particular pharmacophore series, and especially among different pharmacophore series. Simultaneous variations in both k_{on} and k_{off} can therefore result in compounds with similar K_d values but vastly different k_{off} values. Second, the measurement of K_d values alone, without accompanying evaluation of k_{off}, provides no assessment of the temporal components of efficacy (that is, the duration of action) and target selectivity that could be of crucial importance in the open system of *in vivo* pharmacology.

Nature also seems to optimize or ameliorate receptor–ligand occupancy mainly by modulating the specific interactions that influence ligand dissociation. An excellent example of this concept comes from studies of mutation-based resistance to inhibitors of HIV-1 protease. Maschera *et al.*[7] studied mutants of the HIV-1 protease that conferred viral resistance to the AIDS drug saquinavir, in an attempt to offer a biochemical rationale for resistance. These workers found that the *in vitro* values of inhibition constants (K_i) for the wild-type and mutant enzymes, and the corresponding IC_{50} values for inhibition of viral replication in cell culture, were both strongly correlated with the off-rate (k_{off}) of the saquinavir–protease complex (an example of SAR based on K_i values that principally results in the optimization of off-rate; FIG. 1). The association rate constant (k_{on}), by contrast, varied less than twofold among the mutant enzymes. The effects of these mutations on drug–target interactions could therefore be exclusively associated with effects on complex dissociation, and this parameter alone could explain the resistance seen in the mutant virus. A more complete examination of HIV protease–inhibitor interactions was subsequently provided by Shuman *et al.*[8], who studied the values of k_{on}, k_{off} and K_d for amprenavir, indinavir, nelfinavir, ritonavir and saquinavir binding to wild-type and various resistant mutants of the protease. These authors also concluded that:

> "In general resistance was correlated with an increase in the dissociation rate. Resistance resulting from multiple substitutions could arise as a result of decreases in association rate as well as increases in dissociation rate; however, our data suggest that there was no correlation with decreases in association rate above 5×10^6 M^{-1} s^{-1} and resistance."

More recently, the same group studied the interactions of a variety of experimental and clinically used HIV protease inhibitors with the wild-type enzyme[9]. In this study they found that various inhibitors displayed a wide range of both k_{on} and k_{off} values. Neither of these rate constants alone, nor the equilibrium K_d value, correlated well with cellular efficacy in this study, but the authors found that the fraction of free enzyme remaining in the infected cell was highly correlated with the cellular efficacy. Although this latter study highlights the fact that cellular residence time (which is inversely related to free enzyme concentration) might be influenced by factors other than merely the *in vitro* measured value of

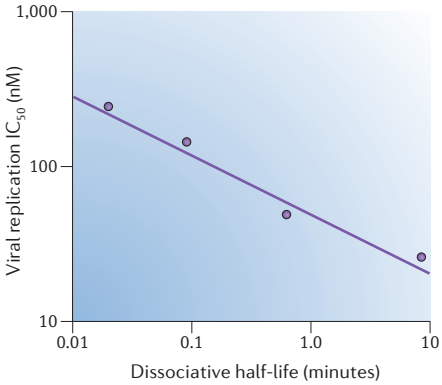

Figure 1 | **Dissociative half-life affects cellular efficacy.** The graph shows the correlation between the dissociative half-life of the complex between HIV-1 protease (wild type and mutants) and an inhibitor of the enzyme (saquinavir), and inhibition of viral replication by saquinavir. Data used to construct this figure were taken from REFS 7,43. IC_{50}, half-maximal inhibitory concentration.

k_{off} (or dissociative half-life), it nevertheless supports the concept that receptor occupancy and duration of occupancy are key determinants of cellular activity.

Impact on target selectivity

During the lead-optimization phase of drug discovery campaigns efforts are commonly focused not only on optimization of target affinity but also on optimization of target selectivity. Target selectivity is a common goal because many adverse clinical events can be linked to collateral binding of drugs to proteins other than the target of interest; such non-target-related toxicity is commonly referred to as off-target or non-mechanism-based toxicity. Target selectivity is typically quantified in closed-system *in vitro* assays as the ratio of the IC_{50} value for the off-target protein over the IC_{50} for the primary target. Such an analysis, however, does not reflect the fact that in the open system of *in vivo* pharmacology selectivity has an important temporal component, as described below.

To exemplify the impact of a drug's dissociative half-life on target selectivity, we consider a case in which an enzyme is the primary *in vivo* target of a drug, and for which there are three other proteins (secondary targets 1–3) to which the inhibitor can bind with varying potencies and corresponding off-rates. One or more of these secondary targets could be serum proteins that non-selectively bind the compound. For our model, we assume that the maximum concentration of the drug in systemic

circulation (C_{max}) is 500 nM, which is achieved 30 minutes following administration, and that the serum half-life of the inhibitor is 60 minutes.

In this theoretical model, the inhibitor (I) binds to its primary target enzyme (E) to form an initial 'classical' EI complex (as in equation 1 above), which over time undergoes isomerization to form a much tighter 'non-classical' complex (EI*; see equations 2 above and 3 below)[4,5], from which the k_{off} is slower than from the EI complex (see equation 3).

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \quad (3)$$

In this case the off-rate of the drug no longer derives solely from the breakdown of the EI complex, but is a more complicated function involving reformation of the EI complex from the EI* complex as it forms over time[5]. Considering that the inhibitor can bind not only to its primary target — its site of biological efficacy — but also to three secondary targets, forming EI and EI* complexes with each binding partner to varying degrees, and working on the assumptions about C_{max} and serum half-life mentioned above, we can model the extent of inhibition of the four targets from the calculated remaining plasma concentration over 10 hours. These values are summarized in TABLE 1.

Note that in this model although the on-rates of the inhibitor for all four targets were fixed to be identical, isomerization to different extents of the four EI complexes to EI* forms results in K_i* and k_{off} values that span three orders of magnitude. The inhibitor effects isomerization to EI* for the primary target, and also for secondary targets 1 and 2 but with minimally increased affinity. The other secondary target, 3, however, remains as a classical EI complex (for which $K_i = K_i$*). This results in the off-rate of the drug from the primary target becoming extremely slow (9×10^{-3} s^{-1}), whereas the off-rates from the secondary targets are rapid (~1 s^{-1}).

Using the calculated serum concentrations of inhibitor at various times after administration, together with the calculated values of K_i, K_i* and k_{off} we can determine the percentages of inhibited enzyme forms (% $(EI + EI^*)/E_{total}$) for each of the four enzyme targets. The results of these calculations are informative, as illustrated in FIG. 2. The serum concentration of the inhibitor rises to 500 nM within 30 minutes, and is diminished to 4% of its C_{max} after 300 minutes. At 420 minutes after administration, 2% of the drug remains in serum and, as a result of the differential inhibition of the four targets to which it binds, nearly 100% of the primary target remains inhibited. In contrast, secondary targets 1–3 are uninhibited to the extents of 15%, 55% and 95%, respectively at this time point. These secondary targets are therefore all reactivated at this point due to metabolic elimination of the drug supply, coupled with the relatively rapid release of the bound drug from these targets. Therefore, the drug in this example is effective and differentially selective at time points when it has been almost completely cleared from systemic circulation. At 600 minutes, despite the almost complete elimination of drug from the serum, the slow dissociation of the inhibitor from the primary target continues to result in its sustained and nearly complete retention of inhibition even at this long time point.

Table 1 | **Calculated inhibition and kinetic constants for a hypothetical drug**

Target	K_i (nM)	K_i* (nM)	k_{off} (s^{-1})	Dissociative $t_{½}$ (s)
Primary	10	0.099	9×10^{-3}	76
Secondary 1	10	1	0.11	6.30
Secondary 2	10	1	1.0	0.69
Secondary 3	100	100	1.0	0.70

The inhibitor is considered to be competitive with the substrates for all targets, and for substrate concentrations that *in vivo* reflect their Michaelis constants. For the kinetic scheme shown in equation 3, $K_i = k_2/k_1$ and $K_i^* = k_2k_4/(k_1(k_3 + k_4))$, $k_{off} = k_2k_4/(k_2 + k_3 + k_4)$; and $t_{1/2} = 0.693/k_{off}$. For all four targets, $k_1 = 10^8$ $M^{-1}s^{-1}$, whereas other rate constants for the four targets were, respectively: k_2 (s^{-1}) = 10, 100, 1, 100; k_3 (s^{-1}) = 1, 1, 0, 0; and k_4 (s^{-1}) = 0.009, 0.11, 1000, 1.

The above model provides a clear sense that the selectivity of the drug is not a static parameter, but is instead time-dependent, and is again determined by the dissociative half-lives for the four enzyme targets. Accordingly, the selectivity of a given drug, as expressed in terms of its temporal binding to a unique or limited number of targets over a period of treatment, is also dependent on the dissociative half-lives from these proteins. As such, the selection of 'lead' compounds based on their dissociative half-lives from primary and secondary targets might be far more relevant for the ultimate efficacy and safety of the compounds than the determination of their IC_{50}, K_d or K_i values at a fixed time point of short duration, as is the current, common practice.

Effects in cellular activity assays

The theoretical example above shows that the dissociative half-life (or off-rate) can uniquely define the duration of efficacy for a drug (see TABLE 2 for some examples of the range of dissociative half-lives exhibited by drugs). Experimentally, there are also cases in which the *in vitro* cellular activity of compounds is associated directly with the dissociative half-life of the receptor–ligand complex. A good example of this comes from the work of Berezov *et al.*[10]. These workers studied a series of peptides that bind to an extracellular domain of the human epidermal growth factor receptor HER2/*neu* and disable the tyrosine kinase activity of the protein. The kinetics of receptor binding and dissociation were measured for nine peptides using surface plasmon resonance technology. The same nine peptides were also tested for their capacity to inhibit cell proliferation in T6-17 cells at a fixed concentration of 1 μg per ml. Berezov *et al.*[10] found that the capacity of the peptides to inhibit cellular proliferation demonstrated a reasonable correlation with the K_d values determined from the ratio of the experimentally measured values of k_{off} and k_{on}. These workers concluded, however, that the cellular percentage inhibition was better correlated with the k_{off} value. The authors of this paper illustrated the data in log–log plots of the cellular percentage inhibition as a function of the various kinetic or thermodynamic constants. However, this method of plotting is sometimes less than optimal for revealing differences in the goodness of fit among data sets. In FIG. 3 we have re-plotted the data of Berezov *et al.* on linear scales, showing the relationship between cellular percentage inhibition and K_d (FIG. 3a), and with the dissociative half-life (FIG. 3b). It is clear from this analysis of the data that the cellular percentage inhibition is most highly correlated with the dissociative half-life. Although the authors offered no theoretical explanation for these observations, the data clearly demonstrated the significant influence of residence time, as measured by the dissociative half-life, on cellular effects of bioactive compounds.

A long dissociative half-life from an intracellular receptor would be expected to translate into sustained efficacy in cell culture after removal of the ligand supply from the extracellular medium. Assays in

which ligand is removed from cell culture and the kinetics of recovery from inhibition of a particular phenotype (such as proliferation) are measured are commonly referred to as 'wash-out experiments'. A recent example of the effects of ligand dissociative half-life on such wash-out recovery comes from the work of Wood *et al.*[11] on inhibitors of epidermal growth factor receptor (EGFR) tyrosine kinase activity. These workers measured K_d values, off-rates and the recovery of cellular proliferation after wash-out for three inhibitors of EGFR that display similar potency in closed-system cellular assays[12]: GW572016 (GlaxoSmithKline), ZD-1839 (AstraZeneca) and OSI-774 (OSI). All three compounds inhibit EGFR by binding to the ATP-binding pocket of the kinase, but display maximal affinity for different conformational states of the enzyme. The dissociative half-lives for all three compounds were determined by recovery of enzymatic activity after dilution, as described in BOX 1 (and in greater detail in REF. 5). For ZD-1839 and OSI-774, rapid recovery of kinase activity after wash-out was observed. The recovery was too rapid to allow the investigators to determine the value of the dissociative half-life, but these experiments put an upper limit on this value of 10 minutes for both compounds. In stark contrast, the recovery of kinase activity after treatment with GW572016 was very slow and yielded an estimated dissociative half-life of 300 minutes for the GW572016–EGFR binary complex. The investigators also measured the recovery of EGFR autophosphorylation activity in HN5 cells after 4 hours of treatment with these three ligands and subsequent wash-out. The OSI-774-treated cells recovered EGFR autophosphorylation activity equal to untreated control cells within 24 hours of ligand removal. Similarly, a significant recovery of EGFR autophosphorylation activity was achieved by removal of ZD-1839, with the cells recovering 60% of control activity within 72 hours of wash-out. Again, GW572016 was distinct from the other two inhibitors in that only 15% of control activity was recovered at 96 hours after compound wash-out.

The data obtained by Wood *et al.*[11] provide a clear example of the extended duration of cellular efficacy that can be achieved with drugs that have long dissociative half-lives. These data also illustrate another common feature of such slow-dissociating ligands that target intracellular receptors: the duration of cellular effects is much longer than would be predicted simply from a consideration of the dissociative half-life of the receptor–ligand complex. In the case of GW572016, the dissociative half-life was measured as 300 minutes. Based on simple first-order kinetics one would expect about 95% of the receptor to be dissociated from ligand after the duration of five half-lives. As such, 24 hours after the wash-out of this ligand, one would expect to have recovered close to 95% of the EGFR autophosphorylation activity of the HN5 cells. Yet the inhibitory effects of the ligand remained in force even 96 hours after compound removal.

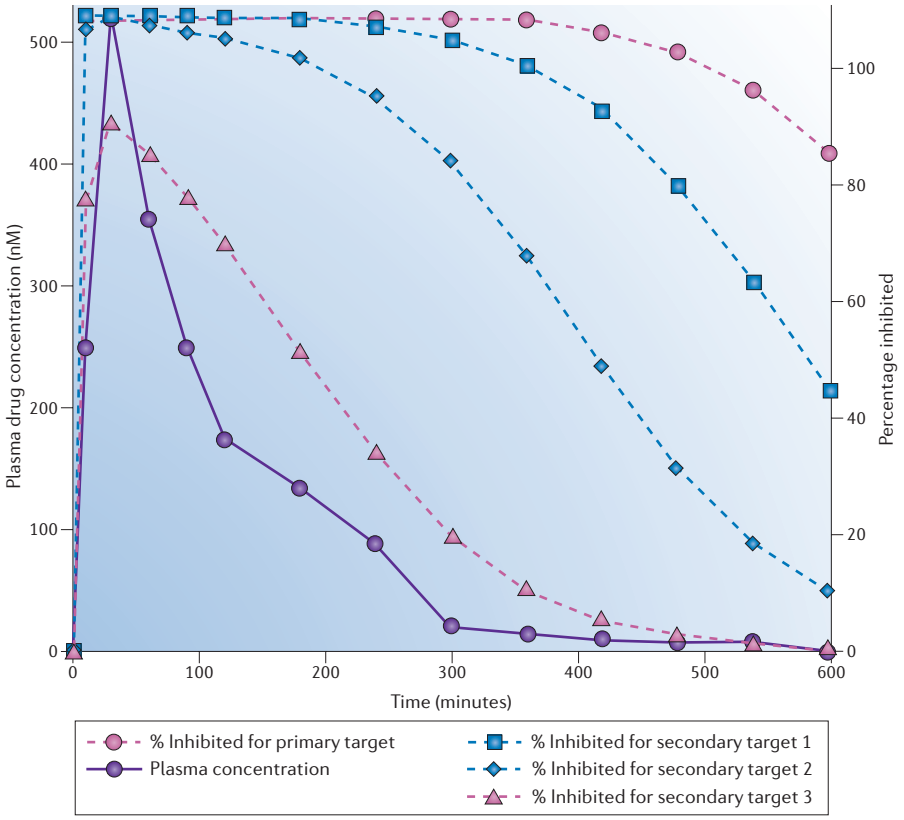

Figure 2 | **Residence time determines target selectivity.** The graph shows plasma drug concentration and percentage inhibition of the primary target and three secondary targets for a hypothetical drug as a function of time after oral administration of the drug. The data plotted here are based on the simulations summarized in TABLE 1.

One explanation for this observation is that the slow dissociation of the ligand from its intracellular receptor creates a high local concentration of ligand in the immediate vicinity of the binding pocket upon dissociation (that is, a proximity effect[13]). Although the bulk of the ligand has been removed from extracellular sources, after dissociation the local concentration remains quite high and might be further augmented by nonspecific interactions with the target protein that maintain a high local ligand concentration for some time. For a typical small-molecule drug targeting a soluble monomeric enzyme, the relative molecular surface area of the small molecule represents ≤3% of that of the target protein[14,15]. Therefore many opportunities for nonspecific encounters between the drug molecule and protein surfaces can be envisaged that might contribute to this proximity effect. Once dissociated, the ligand can diffuse away from the protein target, and eventually out of the cell, or can re-bind to the target macromolecule. Given that re-binding is formally a second-order kinetic process, the rate of re-binding will be significantly accelerated by a high local concentration of ligand. In such a case, the rapidity of re-binding (determined by k_{on} and the local ligand concentration) will greatly out-compete the alternative diffusional process of ligand escape from the cell. In this manner, an extended cycle of slow dissociation and rapid re-binding can ensure that the overall duration of occupancy of the intracellular receptor–ligand complex is extended far beyond what can be measured in cell-free systems (as first suggested in REF 20).

For membrane-associated receptors additional mechanisms for an unexpectedly long duration of inhibition can include receptor desensitization/internalization that are driven by ligand-binding-induced post-translational modification of the target

Table 2 | **Examples of the dissociative half-lives of drugs and drug candidates**

Target	Drug or drug candidate	Dissociative half-life	Refs
Escherichia coli dihydrofolate reductase	Trimethoprim	8 minutes	20
HMG-CoA reductase	Compactin	15 minutes	20
Chicken dihydrofolate reductase	Methotrexate	35 minutes	20
Xanthine oxidase	Allopurinol	5 hours	20
Adenosine deaminase	Deoxyconformycin	40 hours	20
HSP90	Geldanamycin	4.6 hours	39
Human PNP	DADMe-Imm-H	20 minutes	20
Human PNP	DADMe-Imm-G	2 hours	20
COX2	Rofecoxib (Vioxx)	9 hours	40
Viral neuroaminidase	Oseltamivir (Tamiflu)	47 minutes	41
ERBB2/EGFR	Lapatinib	5 hours	11
Human angiotensin II type 1 receptor	Candesartan	1–3 hours	21–23, 42

COX2, cyclooxygenase 2; EGFR, epidermal growth factor receptor; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; HSP, heat-shock protein; PNP, purine nucleoside phosphorylase.

receptor[2,3,16]. Also, in the case of a two-step receptor isomerization mechanism of ligand binding, as shown in equation 2 above, dissociation of the ligand can leave the receptor in an inactive conformation that only slowly relaxes to the active form[2,3]. This phenomenon could also contribute to extending the apparent duration of inhibition for a ligand.

Effects on efficacy and duration *in vivo*

Some of the best-studied examples of the pharmacokinetics and pharmacodynamics of drugs with long dissociative half-lives come from work reported on the 5α-reductase inhibitors epristeride, dutasteride (Avodart; GlaxoSmithKline) and finasteride (Proscar; Merck)[17]. Finasteride is a mechanism-based, reversible inhibitor of steroid 5α-reductase that is used clinically to treat benign prostate hyperplasia (BPH). By inhibiting 5α-reductase, finasteride blocks the production of the male hormone dihydrotestosterone (DHT) from its precursor testosterone. The accompanying reduction in systemic levels of DHT is beneficial in treating not only BPH, but also male-pattern baldness and, potentially, acne. Finasteride functions through an unusual mechanism of action, forming a covalent adduct with the NADPH co-substrate within the active site of the enzyme[18]. The resulting inhibitory NADP–dihydrofinasteride complex inhibits the enzyme with a K_i of approximately 0.3 pM. This tight-binding inhibitor is formally reversible, but attempts to measure the rate of dissociation of the inhibitor complex from the enzyme have yielded only a lower limit on the half-life of the inhibited enzyme species of >30 days. For all practical purposes, then, the NADP-dihydrofinasteride covalent adduct is an irreversible inhibitor of steroid 5α-reductase.

At single oral doses of 5–100 mg, finasteride achieved its peak plasma concentration between 1.8 and 2.8 hours in humans[19]. The systemic level of the compound then declined, with a half-life of 4.7–7.1 hours (there was no systematic relationship between dose and half-life). At all doses, therefore, the compound had reached very low trough levels in circulation by 24 hours after dosing. In contrast to these pharmacokinetic data, the drug had a prolonged effect on serum levels of DHT after a single oral dose. At all doses, a significant decrease in serum DHT levels was observed within 4 hours of oral administration, and the DHT levels remained suppressed for >72 hours (FIG. 4). In patients receiving multiple doses of 1 or 10 mg of finasteride once daily for 7 days, the serum levels of DHT remained significantly suppressed, relative to placebo, for up to 7 days after administration of the final dose. The pharmacological effects of finasteride action therefore endure for a significant time after circulating levels of the drug have been cleared by normal metabolic processes. The extended effects of finasteride have been explained by the extremely long residence time of the inhibitory complex on the target enzyme. In fact, the recovery of circulating DHT levels after 72 hours has been suggested to reflect the half-life of new enzyme synthesis, rather than the dissociation of the inhibitory complex from the enzyme. It is widely assumed that the clinical utility of finasteride stems from its long residence time on the steroid 5α-reductase enzyme, as reflected in its extremely long dissociative half-life.

A second example of this concept comes from the recent work of Schramm and co-workers[20] on the design of transition-state analogue inhibitors of the enzyme purine nucleoside phosphorylase (PNP). PNP is a target for several autoimmune diseases. Schramm and coworkers have used the X-ray crystal structure of the enzyme together with a sophisticated set of experimental kinetic and computational methods to design small-molecule inhibitors that would optimally mimic transition-state interactions with the enzyme active site. One of the compounds resulting from these efforts is DADMe-immucillin-H. This compound displays slow, tight-binding inhibition of PNP with an overall K_d value of 16 pM. The off-rate of the PNP–inhibitor complex is slow, yielding a dissociative half-life of ~20 minutes. As described above, these authors speculated that the duration of occupancy of the compound on the target enzyme is greatly extended by the cumulative effects of the slow dissociation rate of the binary enzyme–inhibitor complex and the facile re-binding to the enzyme within the intracellular milieu. Oral dosing of mice with this compound resulted in a significant diminution of circulating PNP enzyme activity, reaching maximal effect about 30 minutes after dosing. Recovery of PNP enzyme activity occurred quite slowly after a single oral dose of the compound. The half-life for recovery of circulating PNP enzyme activity has been measured to be 275 hours (11.5 days). This value correlates well with the expected half-life for new erythrocyte synthesis in mice (~12.5 days), suggesting that the recovery of enzymatic activity is not due to compound dissociation from its target, nor compound metabolism, but rather from *de novo* synthesis of new enzyme by the organism. Schramm and co-workers point out that inhibition that is sustained until cellular replacement of the target by new protein synthesis represents the ultimate physiological efficacy that can be attained by a therapeutic agent. They have coined the phrase 'ultimate physiological inhibitors' to describe drugs that demonstrate this effect. Optimization of compound residence time, by minimization of the dissociation rate constant, seems the most practical way to systematically approach this goal through medicinal chemistry efforts.

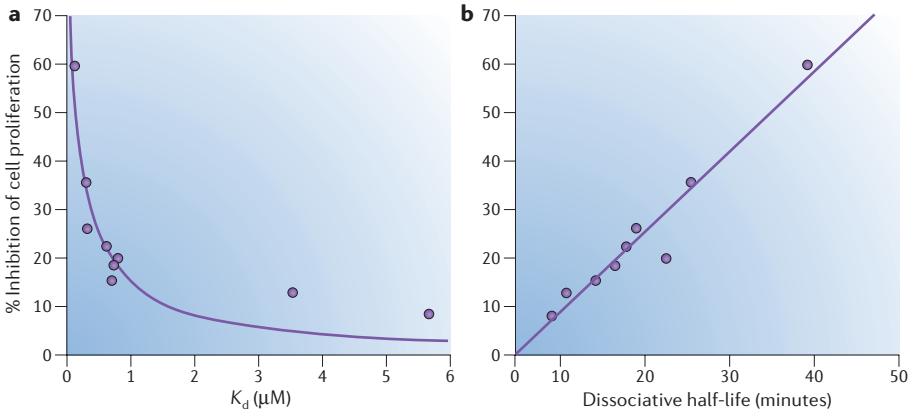

Figure 3 | **Relating cell growth inhibition to receptor–ligand interactions. a** | The correlation between the equilibrium dissociation constant (K_d) of different peptide-HER2/*neu* receptor complexes and the percentage inhibition of proliferation in T6-17 cells caused by addition of 1 μg per ml of each peptide. The line drawn through the data represents the expected behaviour based on a standard concentration–response isotherm[5]. **b** | The correlation between the dissociative half-life of the peptide–HER2/*neu* receptor complexes and the percentage inhibition of T6-17 cell proliferation caused by addition of 1 μg per ml of the peptides. The line drawn through the data represents the least-squares best fit to a simple linear equation. Data used to construct this figure were taken from REF. 10.

A final example is provided by the comparisons of the pharmacokinetics and pharmacodynamics of the angiotensin II type 1 receptor (ATR1) antagonists losartan (Cozaar; Merck) and candesartan (Atacand; AstraZeneca)[21–23]. Blockade of this receptor interferes with the renin–angiotensin system, resulting in lowered blood pressure. ATR1 antagonists are effective in treating hypertension, similar to angiotensin-converting enzyme (ACE) inhibitors, but without the cough and angio-oedema adverse reactions associated with the latter class of drugs. Losartan was the first example of an ATR1 antagonist to enter the clinic and candesartan was subsequently identified as a potent, long-lasting antagonist of the receptor. Comparison of the *in vitro* pharmacology of the two antagonists determined that candesartan demonstrates tighter binding to the receptor than losartan (K_d: ~7 nM versus ~350 nM) and a significantly longer dissociative half-life (66–152 minutes for candesartan versus 5 minutes for losartan). *In vitro* binding studies suggest that the long dissociative half-life of candesartan relates to a two-step receptor isomerization mechanism of binding[23].

The long dissociative half-life of candesartan translates into a prolonged capacity to block the response to angiotensin II in isolated rat portal vein preparations[24]. These preparations were treated for 60 minutes with losartan or candesartan and then washed repeatedly. The time required to recover 20% and 60% of control responses to angiotensin II was then determined. For the losartan-treated preparation it took 6 and 18 minutes to recover 20% and 60% response, respectively. By contrast, the candesartan-treated preparations took 72 minutes to recover 20% response and did not recover 60% response within 2 hours. The duration of action of candesartan in tissue seems to be extended both by its long dissociative half-life and by significant drug re-binding to its receptor. Vanderheyden *et al.*[23] looked at the dissociative half-life for candesartan binding to ATR1 expressed in Chinese hamster ovary cells. When cells pre-incubated with [^{3}H]candesartan were washed with medium alone, the observed dissociative half-life was found to be 462 minutes. When, however, the cells were washed with medium containing either 10 μM losartan or 0.1 μM cold candesartan, the dissociative half-life was reduced to ~100 minutes; the presence of the cold antagonists in the wash medium prevented significant re-binding of the radiolabelled drug. The authors suggested that the long dissociative half-life and re-binding of candesartan to the receptor in tissue probably accounts for the prolonged blood-pressure lowering seen in humans with this drug, relative to that seen for losartan.

A comparison of the efficacy and duration of action for candesartan and losartan was made in hypertensive patients by assessing clinic and ambulatory blood pressure[22]. Doses of 16 mg of candesartan and 100 mg of losartan once daily both resulted in significant lowering of mean sitting blood pressure (both systolic and diastolic), relative to placebo, 24 hours after dosing. At 48 hours after dosing, however, the blood pressure of patients treated with losartan were no longer significantly different from that of those treated with placebo, whereas those treated with candesartan continued to demonstrate significantly lower blood pressure. Mean reductions in ambulatory systolic and diastolic blood pressure were measured over a 36-hour period after 4 weeks of dosing with each drug. A 16 mg per day dose of candesartan produced a significantly greater reduction in systolic blood pressure than did 100 mg per day of losartan over the first 24-hour period. Likewise, over the 0–36-hour monitoring period, the 16 mg per day candesartan treatment reduced both systolic and diastolic blood pressure to a significantly greater extent than did 100 mg per day of losartan. Most importantly, the mean systolic and diastolic ambulatory blood pressure remained significantly lower with candesartan relative to losartan treatment during a day of a missed dose. Candesartan therefore demonstrates a much longer duration of action than does losartan. The sustained clinical action of candesartan is suggested to result from the much longer dissociative half-life of the candesartan–ATR1 complex[22].

Mitigating factors

Although maximizing target residence time is clearly a method for drug optimization, there are factors that limit the ultimate utility of this approach. One factor, mentioned above, is that eventually the efficacy of drugs with a long residence time will be dissipated through re-synthesis of new molecules of the target receptor by the organism. Although this scenario represents 'ultimate physiological inhibition', the dosing interval required for effective drug action will nevertheless be dictated by the rate of new protein synthesis. For infectious disease targets, viral and bacterial replication rates can be quite rapid, thereby minimizing the effectiveness of drugs with long residence time as a mechanism for extended pharmacological effect. Similarly, for targets that are rapidly synthesized and rapidly degraded at key points in the cell cycle, the impact of a long residence time might be diminished; however, the lack of cell-cycle synchronization in, for example, cancerous tumours might offset this mitigating factor. These limitations notwithstanding, it still seems to us that maximizing the residence time of a drug will provide the optimal efficacy *in vivo*.

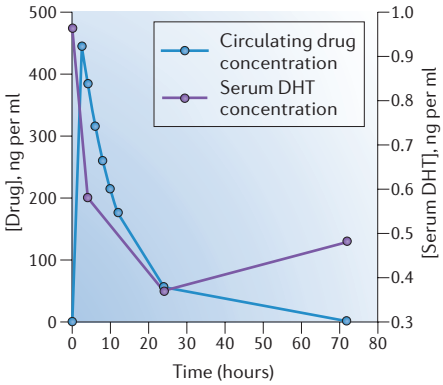

Figure 4 | **Extended action of finesteride.** Idealized pharmacokinetics (circulating drug concentration), based on reported C_{max}, t_{max} and $t_{½}$ and pharmacodynamics (serum DHT concentration) after a single oral dose (50 mg) of finasteride to healthy volunteers. Data used to construct this figure were taken from REF. 19 and references therein.

There might, however, be specific cases in which a long residence time might be detrimental to drug development because of safety considerations. Slow dissociation of drugs from a target receptor is often associated with a ligand-binding-induced conformational change of the receptor that 'locks' the compound into a binding-pocket configuration that is optimized for favourable interactions[5]. For cell-surface targets, particularly in systemic circulation, the altered conformation of the receptor that is induced by tight-binding drugs could expose cryptic epitopes that can be recognized by the immune system as 'foreign', and thereby elicit an immune response. A drug with a long residence time on such a receptor would increase the duration of antigen presentation, and therefore increase the likelihood of an immune response. This situation was encountered with roxifiban (DuPont), a tight-binding, slowly-dissociating antagonist of the platelet-surface receptor glycoprotein IIb/IIIa[25]. Binding of the compound induces an alteration in the receptor conformation on the platelet surface. The altered conformation presents neoepitopes referred to as 'ligand-induced binding sites'. The induction of these neoepitope sites could be monitored *in vitro* by gel-shift analysis with monoclonal antibodies that specifically recognized the altered conformational state of the receptor. *In vivo*, antibody recognition led to an immune-based clearing of platelets, resulting in severe thrombocytopaenia with a frequency of about 2% of patients. This side effect was severe enough to halt further development of roxifiban[26,27]. Although recognition of a drug-induced target conformation by the immune system could be a significant issue for clinical development, this is less likely to be a concern for the majority of drug targets that are either intracellular or presented on the surface of non-circulating cells.

A second situation in which a long residence time might be detrimental to safety is when an adverse effect is due to pharmacology resulting directly from formation of the receptor–ligand complex. In this perspective we have highlighted the advantages of a long residence time for potentially mitigating toxicities due to collateral drug binding to proteins other than the target receptor; such toxicity is commonly referred to as off-target toxicity. Toxicity can also result from target receptor engagement by a drug. In this latter on-target-toxicity case, differentiation of beneficial pharmacology from toxicity can be very challenging. In some cases, however, there might be a temporal distinction between desired effect and toxicity, such that rapid binding and dissociation of a ligand results in the desired pharmacology[3], whereas sustained residence of the ligand on the receptor leads to adverse events. This has been suggested to be the case for D_2 receptor blockers that act as atypical antipsychotics[28]. Kapur and Seeman[28] measured the values of K_d, k_{off} and k_{on} for a collection of typical and atypical antipsychotic drugs and found that a rapid dissociation rate was the only feature that distinguished the atypical drugs from the typical antipsychotics. However, this explanation is not universally accepted[29,30]. Other workers suggest that the atypical behaviour of these drugs stems from the spectrum of binding affinity of these compounds for different receptors. They argue that it is low affinity for the D_2 receptor, not the rapid off-rate *per se*, that results in atypical behaviour. Likewise, it has been argued that channel blockers demonstrating rapid on- and off-rates from the *N*-methyl-D-aspartate receptor (NMDAR) can provide protection against glutamate-mediated cell death in cerebral ischaemic diseases, while sparing the positive effects of receptor action on cognitive function and avoiding the typical side effects caused by hallucinations and drowsiness of high-affinity antagonists[31,32]. Lipton and Chen[32] suggest that the optimal pharmacology would derive from uncompetitive open-channel blockers with a rapid off-rate from the receptor. By binding only to the open-channel conformation (uncompetitive antagonism), such compounds would selectively act in the presence of excessive channel activity. By dissociating rapidly from the receptor, the compounds would avoid progressive blockade of normal synaptic transmission by limiting the accumulation of bound, open channels. Again, the relationship between this differentiation of positive and negative effects of NMDAR antagonists with rapid on- and off-rates is hypothetical and requires additional study. Nevertheless, these examples represent a theoretical situation in which a long residence time for a receptor–ligand complex would be contraindicated.

Taken to the extreme, the benefits of a slow dissociation rate for a receptor–ligand complex could lead one to speculate that the ultimate in drug efficacy and duration of action would come from compounds that irreversibly inactivate their target receptor by forming a covalent adduct (and therefore never dissociating). Indeed, there are numerous examples of drugs used in human medicine that act through such a mechanism (aspirin, for example). However, the suitability for therapeutic use of reactive compounds that form covalent adducts with protein targets is very dependent on the specific nature of the chemistry of covalent adduct formation. Inactivators that are generally unreactive until bound to and acted on by the active site of an enzyme are referred to as 'mechanism-based inactivators'. Because these compounds rely on the *in situ* chemistry of enzyme catalysis to form a covalent adduct, they typically display high degrees of target specificity and are therefore appropriate for use as drugs. By contrast, compounds that contain electrophilic moieties that display general reactivity with protein-based nucleophiles are generally too nonspecific to be of use in human medicine (but there are notable exceptions to this generality, especially in the fields of antibacterial and cancer chemotherapy). The advantages and disadvantages of various mechanisms of covalent adduct formation in the field of drug discovery have recently been reviewed elsewhere[5]; the interested reader is referred to this text and to the references therein.

Conclusions

The examples presented in this perspective make a strong case for the value of measuring the dissociative half-life of receptor–ligand complexes, and the use of this parameter for compound optimization during drug discovery efforts. Most of our focus has been on the extended duration of drug action that can be achieved *in vivo* with long-residence-time drugs. By maximizing the dissociative half-life one can approach 'ultimate physiological inhibition',

Glossary

Association rate constant/on-rate/k_{on}
A second-order rate constant that quantifies the rate at which a free ligand and free receptor combine (through collisional encounters) to form a binary receptor–ligand complex.

Closed system
A system in which the binding partners (ligand and receptor) are present at temporally invariant concentrations throughout the course of an experimental measure.

C_{max}
The maximum concentration of drug in systemic circulation that is attained after dosing. The C_{max} is typically attained at a specific time point after dosing and this time point is commonly referred to as the t_{max}.

Dissociation rate constant/off-rate/k_{off}
A first-order rate constant that quantifies the rate at which a binary receptor–ligand complex dissociates to the free ligand and free receptor.

Equilibrium model (of drug action)
A model in which the pharmacological activity of a drug is dependent on the establishment of an equilibrium between the free concentration of drug and the concentration of drug bound to its pharmacological target receptor.

Equilibrium dissociation constant (K_d)
An inverse measure of the affinity of a receptor–ligand pair under equilibrium conditions. The value of K_d is mathematically equivalent to the ratio k_{off}/k_{on}.

Inhibition constant (K_i)
The K_i is the equilibrium dissociation constant for an inhibitor and a specific form of the enzyme target. See REF. 5 for further information.

Ligand
For the purposes of this review, the term ligand refers to a small molecule, such as a drug, that binds to, and thereby affects the activity of, a specific macromolecular target (receptor).

Non-equilibrium model (of drug action)
Any model in which the pharmacological activity of a drug is most dependent on a departure from equilibrium conditions with respect to the drug and its pharmacological target receptor. See REFS 4,5 for additional information.

Open system
A system in which the concentration(s) of one or more of the binding partners (ligand and receptor) can change over time as molecules of these species enter the system (for example, by distribution to the tissue) and leave the system (for example, by metabolism and other elimination mechanisms).

Receptor
A biological macromolecule responsible for a specific physiological activity that is the target of binding for a particular drug or other ligand.

Binary complex residence time
The amount of time that a ligand resides on a given receptor after binding. Note that this is the definition of residence time used in this article; the term residence time has additional, unrelated meanings (for example, tissue residence time and so on) in other aspects of pharmacology.

IC_{50}
The concentration of a substance that results in a 50% effect on some measure of biochemical function or substance–target binding interaction.

Receptor–ligand complex
(RL). The binary complex between a given receptor and a given ligand. Drug–target complexes are referred to throughout this review as receptor–ligand complexes, regardless of the specific chemical and biochemical nature of the binding partners (for example, enzyme–inhibitor complexes, G-protein coupled-receptor–antagonist complexes, ribosome–antibiotic complexes and so on). In cases of two-step binding mechanisms, RL refers to the initial encounter complex between the receptor and the ligand. In the review an example of a two-step binding mechanism is presented in which the receptor is an enzyme and the ligand is an inhibitor. In such a case the RL complex is more specifically referred to as EI.

RL*
A very high-affinity binary complex between a receptor (for example, an enzyme) and ligand (for example, an enzyme inhibitor) that usually results from isomerization of the receptor (that is, a conformational change) subsequent to initial binding of the ligand to the receptor. In the case of enzyme inhibitors, the initial encounter complex between the enzyme and inhibitor is referred to as EI and the subsequent high-affinity complex is referred to as EI*. See REFS 5,36 for further information.

Structure–activity relationship
(SAR). This term refers to the relationship between the molecular structure of a ligand and its affinity and/or effect on a specific receptor.

in which recovery from inhibition only occurs as the result of new target synthesis by the cell or organism. Systematic use of the dissociative half-life as a metric during SAR studies could provide a new dimension to medicinal chemistry efforts towards residence-time optimization. Of course, *in vivo* efficacy and duration of action are affected by multiple variables beyond the biomolecular interactions of a ligand with its receptor. We do not suggest that *in vitro* measurement of the dissociative half-life will suffice to predict the cellular and *in vivo* efficacy of compounds in general. Rather, the use of dissociative half-lives provides an additional parameter for compound optimization, in concert with optimization of pharmacological parameters such as absorption, cytochrome P450-mediated metabolism, serum protein binding and the like. The richer texture of the SAR afforded by assessment of dissociative half-lives can, in favourable cases, provide insights to medicinal chemists that might not be obvious from the more traditional metrics of target affinity, such as IC_{50} values.

Perhaps equally important is the potential to ameliorate off-target-based toxicities with such drugs, because of their capacity to limit the concentration and duration of systemic exposure required for pharmacodynamic efficacy[5]. This, however, is less predictable from *in vitro* studies alone.

Much of what is presented here is speculative as there is a paucity of concrete examples that can be drawn on from the literature. This is largely a reflection of the heavy reliance of drug researchers on the thermodynamic dissociation constant, K_d (or K_i), as the primary measure of drug–target potency. Until now, few researchers have made the effort to systematically study compound SAR on the basis of on-rate and/or off-rate determinations, and fewer still have translated this information into *in vivo* effects. There are today several convenient experimental methods for determining the off-rate of receptor–ligand complexes *in vitro* (BOX 1). All of these methods are easily performed in any modern biochemistry laboratory. Although literature reports of receptor–ligand off-rates have been sparse up to now, there is today no substantial experimental barrier to obtaining these rate constants. It is our hope that the present review will stimulate drug discovery scientists to invest more in measuring this important determinant of drug action.

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Competing interests statement

The authors declare competing financial interests: see Web version for details.

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CORRIGENDUM

Drug–target residence time and its implications for lead optimization

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The authors wish to correct values in Table 1 and the footnote (page 733). A number of the values in the Table and in the footnote are incompatible with the corresponding data in Figure 2 (page 734). Although these errors affect the quantitative values reported in Table 1, they do not impact on the overarching perspective nor the conclusions drawn in this article.

The corrections for the Table and the footnote should read:

Table 1 | **Calculated inhibition and kinetic constants for a hypothetical drug**

Target	K_i (nM)	K_i* (nM)	k_{off} (s^{-1})	Dissociative $t_{½}$ (s)
Primary	10	0.099	9×10^{-3}	76
Secondary 1	1	0.4	0.2	3.10
Secondary 2	10	3	0.4	1.60
Secondary 3	50	50	1.0	1.00

The inhibitor is considered to be competitive with the substrates for all targets, and for which substrate concentrations *in vivo* reflect their Michaelis constants. For the kinetic scheme shown in equation 3, $K_i = k_2/k_1$ and $K_i^* = k_2k_4/(k_1(k_3 + k_4))$, $k_{off} = k_2k_4/(k_2 + k_3 + k_4)$; and $t_{1/2} = 0.693/k_{off}$. For all four targets, $k_1 = 10^9\ M^{-1}s^{-1}$, whereas other rate constants for the four targets were, respectively: k_2 (s^{-1}) = 10, 1, 10, 50; k_3 (s^{-1}) = 1, 0.75, 1, 0; and k_4 (s^{-1}) = 0.01, 0.5, 0.5, 1.0.