



Molecular determinants of drug–receptor binding kinetics

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It is increasingly appreciated that the rates at which drugs associate with and dissociate from receptors — the binding kinetics — directly impact drug efficacy and safety. The molecular determinants of drug–receptor binding kinetics remain poorly understood, however, especially when compared with the well-known factors that affect binding affinity. The rational modulation of kinetics during lead optimization thus remains challenging. We review some of the key factors thought to control drug–receptor binding kinetics at the molecular level — molecular size, conformational fluctuations, electrostatic interactions and hydrophobic effects — and discuss several possible approaches for the rational design of drugs with desired binding kinetics.

Introduction

The receptor theory of drug action posits that a drug works only when bound to its target receptor [1]. Direct measurement of the extent to which a drug is bound to its receptor at equilibrium — the binding affinity — was, however, not possible until long after the theory was first postulated. Accordingly, drug discovery programs historically sought to optimize drug efficacy, not affinity, usually in the context of whole cells, tissues or animals. Only with the advent of identifiable, and ultimately purifiable, molecular receptors that enabled the direct measurement of binding affinity did optimization of binding affinity guide most early-stage discovery efforts.

This emphasis on binding affinity — quantified either as K_d , the equilibrium dissociation constant, or its proxies, IC_{50} or EC_{50} , the drug concentrations giving half-maximal inhibition or effect — is predicated on the assumption that affinity is an appropriate surrogate for *in vivo* efficacy. Although many highly efficacious drugs have been discovered on that basis, recent studies have shown that the kinetics of drug–receptor binding could be as important as, and in some cases more important than, affinity in determining drug efficacy [2–4]. In an open, *in vivo* system the concentration of the drug varies over time — potentially on timescales faster than

binding and unbinding to its receptor — such that binding equilibrium might not be reached or maintained; for some drugs, attainment of equilibrium might not even be desirable. In these cases, equilibrium binding affinity is no longer an appropriate surrogate for efficacy — instead, the rates of drug–receptor association and dissociation, as reflected by the rate constants k_{on} and k_{off} , are more appropriate (Box 1).

The concepts underlying rational optimization of binding affinity are relatively well understood, but the same is not true for binding kinetics. Much less is known about the molecular determinants of binding kinetics than about those of binding affinity. A major challenge with optimization of kinetics is the fundamental difficulty in characterizing transient states. Binding affinity depends on the free energy difference between the bound and unbound states, both of which are stable and generally easily observable. On- and off-rates depend instead on the height of the (highest) free energy barrier separating those states, yet the atomic arrangement of the drug and the receptor at this point of highest free energy — the transition state — has only a fleeting existence (Fig. 1a). Understanding the molecular interactions between drug and receptor at this difficult-to-observe transition state (Box 1) is thus central to the rational control of drug binding kinetics.

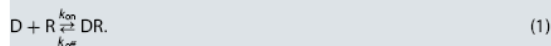
Despite these challenges, the intentional and rational optimization of k_{on} or k_{off} opens up a new, temporal dimension for

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BOX 1

Binding kinetics primer

Consider a simple binding reaction between a drug (D) and a receptor (R) to form a bimolecular complex (DR):



This two-state mechanism comprises a single elementary step — the binding (or unbinding) of the drug — without any intermediate states; it suffices to illustrate several key points although drug binding often involves one or more intermediates [4].

First, the binding affinity is described by the dissociation constant, K_d , a ratio of the relevant concentrations at equilibrium:

$$K_d = \frac{[D][R]}{[DR]}. \quad (2)$$

At equilibrium, the drug concentration at which half the receptor binding sites are occupied is equivalent to K_d (both typically measured in units of mol/L); K_d is directly related to the free energy difference, ΔG_d , between the bound and unbound states (Fig. 1a). In contrast to K_d , which is determined solely by stable molecular interactions between the drug, receptor and solvent, the rate constants k_{on} and k_{off} depend upon transient interactions along the binding pathway, particularly the highest free energy barrier — the transition state — that separates the bound and unbound states (Fig. 1a). The thermodynamics and kinetics of binding are linked as:

$$K_d = \frac{k_{\text{off}}}{k_{\text{on}}}. \quad (3)$$

Second, the free energies of the unbound and bound states, and of the transition state, can, in principle, be varied independently of one another (Fig. 1a). Destabilizing only the transition state

decreases both rates without altering affinity; conversely, stabilizing the transition state increases rates. Destabilizing the bound state weakens affinity and increases the off-rate without altering the on-rate, whereas altering the energy of the unbound state affects the on-rate and the affinity only. In practice, however, achieving any of these 'corner cases' — the 'pure' alteration of just two of the constants K_d , k_{on} and k_{off} without affecting the third — is difficult. Notably, changes in k_{off} can result in no measureable effect on K_d if there are compensatory changes in k_{on} . In such a case, ligand optimization based on affinity alone would hide the molecular determinants of binding kinetics behind an unchanging K_d . Indeed, QSAR models that resolve k_{on} and k_{off} can be more reliable than those based on binding affinity alone [56,57].

Third, the average time that a drug stays bound to its receptor, the residence time, $t_R \equiv 1/k_{\text{off}}$, and similarly the half-life, $t_{1/2} \equiv \ln 2/k_{\text{off}}$, are useful measures of binding kinetics. In a two-state mechanism, for instance, a typical nanomolar affinity drug that binds at the maximal, diffusion-limited on-rate ($k_{\text{on}} \approx 10^9 \text{ M}^{-1} \text{ s}^{-1}$) [2,58] would have a residence time of $\sim 1 \text{ s}$. Importantly, t_R and $t_{1/2}$ are independent of drug concentration. Thus, even when the free concentration of a long-residence-time drug falls below the K_d , a significant population of receptors will still be occupied by drug.

Finally, the on- and off-rates represent elementary mechanistic steps — described by the simple kinetic constants k_{on} and k_{off} — only for a two-state mechanism. In more common, non-two-state mechanisms (e.g. a three-state mechanism; Fig. 1a) the apparent rate constants are composites of multiple elementary rate constants that describe the transitions between unbound, intermediate and bound states. Such a composite nature — indeed, the mere existence of a non-two-state mechanism — often reveals itself in counterintuitive binding kinetics [59].

controlling drug behavior that has important therapeutic implications for drug efficacy and drug safety. The residence time of a drug–receptor complex, $t_R \equiv 1/k_{\text{off}}$, is often a better predictor of efficacy than binding affinity is (Fig. 1b) [2,4,5]. Similarly, when achieving target selectivity is important, a drug with a longer residence time on one receptor can select kinetically for that receptor over another, even when the affinity for both receptors is comparable [2]. Conversely, drugs with faster dissociation rates can increase the therapeutic index (the key measure of drug safety, defined as the ratio of a drug's toxic dose to its efficacious dose) when extended, non-physiological drug occupancy of the target receptor causes toxicity [6–8]. Finally, a faster-binding drug might target a short-lived receptor more effectively [9].

Here, we review the key molecular determinants currently known to control drug–receptor binding kinetics. Although the various interactions that influence the kinetics are often coupled, we discuss each determinant separately, citing examples in which each dominates. After briefly discussing recent advances in methods to elucidate these molecular determinants, we conclude by speculating on how these insights might be used to design drugs rationally with desired binding kinetics.

Molecular determinants of binding kinetics

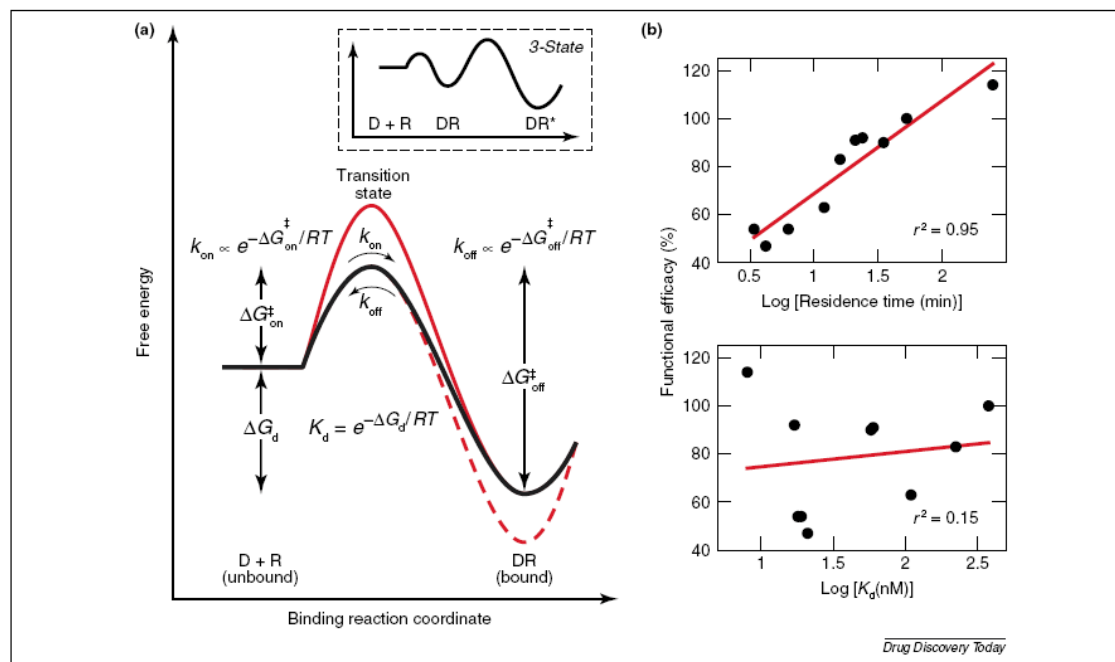
Binding site accessibility and drug size

Intuitively, drug binding speed must be governed, in part, by accessibility of the receptor binding site: limited access through a narrow passageway should be inherently slower than unimpeded

access to, or egress from, an open binding site. The larger the drug, the more this simple notion should apply. Indeed, a survey of over 2000 drugs binding to G-protein-coupled receptors (GPCRs), protein kinases and other enzymes found that higher molecular weight drugs tend to have lower off-rates (i.e. longer residence times), independent of confounding variables such as clogP [10]. One subtlety is that it can be difficult to disentangle the molecular determinants that decrease k_{off} from those that decrease K_d (which is simply $k_{\text{off}}/k_{\text{on}}$; Box 1; Fig. 1a). That is, this correlation of residence time with molecular weight can reflect either the known correlation of molecular weight with affinity (i.e. bound-state stabilization) or a correlation with barrier height (i.e. transition-state destabilization). Nevertheless, the intuitive dependence of binding speed on size is a real effect for certain receptors, particularly for those with deep binding pockets.

Conformational fluctuations

Drugs and receptors are dynamic and their conformational flexibility can impact binding kinetics, sometimes in subtle ways. In the same sample of over 2000 drugs, for instance, longer ligand residence time was also correlated with a greater number of rotatable bonds [10]. Similarly, restricting ligand conformational flexibility accelerated the binding of a series of corticotropin-releasing factor type 1 receptor antagonists [11]. The active conformation of these antagonists comprises two orthogonally disposed aromatic rings. Substitution of one of the rings with a 2-chloro group, to enforce that orthogonality, increased the binding

**FIGURE 1**

Drug affinity and binding rates depend on the free energy profile of binding. (a) A simple free energy profile (black) of a drug (D) binding to a receptor (R) to form a drug-receptor complex (DR). The free energy difference between the bound and unbound states, ΔG_d , determines the binding affinity. The association and dissociation rate constants, k_{on} and k_{off} , depend on the free energy differences, $\Delta G_{on}^\ddagger$ and $\Delta G_{off}^\ddagger$, between these states and the transition state. Here, R is the ideal gas constant and T is the temperature. Modulations of the free energy profile that result in the same decrease in k_{off} are shown in red; the solid red profile achieves this decrease by increasing the barrier height (i.e. destabilizing the transition state), whereas the dashed red profile does so by increasing the affinity (i.e. stabilizing the bound state). These profiles represent two extremes of binding kinetics modulation. In practice, changes in binding kinetics will probably involve a combination of stabilization or destabilization of the bound state and the transition state. (b) Drug residence time, not affinity, predicts functional efficacy of a series of agonists of the A_{2A} adenosine receptor (data from [5]). Residence time (top panel) is highly correlated with functional efficacy (determined using a label-free whole-cell assay), but there is little correlation with binding affinity (bottom panel). For these compounds k_{off} was only poorly correlated with K_d , and the values of k_{on} vary by over 300-fold.

rate 33-fold, increased the affinity 63-fold and left the off-rate more or less unchanged.

Receptor flexibility often plays an important part in modulating the binding kinetics of buried or occluded binding sites. Early studies on carbon monoxide (CO) unbinding from myoglobin revealed the importance of protein breathing motions in enabling CO escape [12]. Indeed, rigidification of myoglobin with an engineered disulfide bond slows CO dissociation [13].

In the binding of more-drug-like molecules receptor flexibility can take the form of intricate loop motions [14–17]. Binding of inhibitors to InhA, the enoyl-ACP reductase of *Mycobacterium tuberculosis*, for instance, involves conversion of a disordered loop into an ordered α helix at the active site; this large conformational change appears to impact the rate of inhibitor binding and unbinding significantly, with slow-onset and slow-offset inhibition being associated with the ordered helix (Fig. 2a). Luckner and co-workers [14] thus prepared a triclosan-like inhibitor, PT70, by the addition of a single methyl group specifically designed to interact with the ordered InhA helix and the NAD^+ cofactor. This simple change was found to increase the

residence time of PT70 by several orders of magnitude compared with triclosan itself.

Similarly, certain desirable features of tiotropium that have contributed to its clinical success in the treatment of chronic obstructive pulmonary disease might be attributed to conformational flexibility of a muscarinic receptor extracellular loop [15]. The antagonist tiotropium has an extraordinarily long residence time on the M3 muscarinic receptor ($t_R \approx 30$ hours), substantially longer than that for other receptor subtypes such as M2 ($t_R \approx 3$ hours); once-daily dosing thus covers the M3 target without prolonged, deleterious inhibition of M2. This kinetic selectivity of tiotropium appears to increase its therapeutic index, despite comparable binding affinity for M2 and M3 [18] (for an alternative hypothesis see recent work by Charlton and co-workers [19]). In molecular dynamics simulations of both receptor subtypes [15], we found that extracellular loop 2 (ECL2) of M2 is more flexible than ECL2 of M3: M2 ECL2 was found more often in an open conformation that might enable easier ligand egress from the binding site (Fig. 2b). This difference in ECL2 loop dynamics could explain the 10-fold longer residence time of tiotropium on M3.

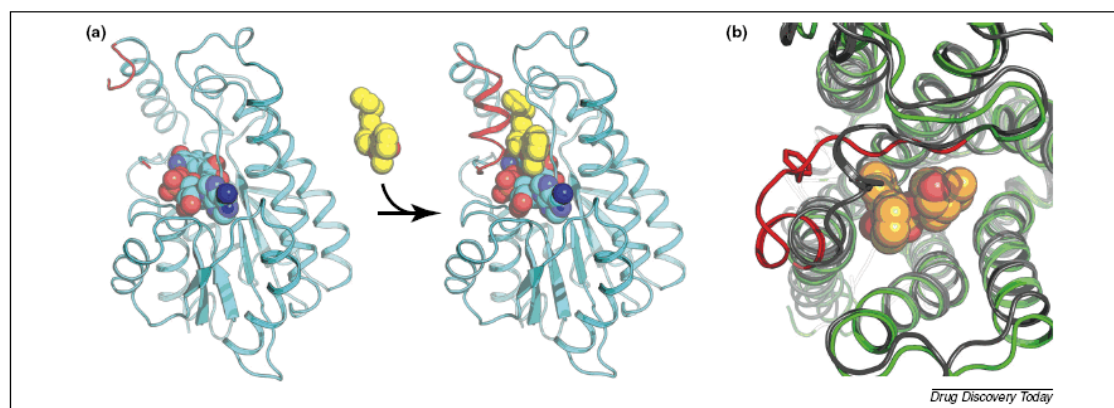


FIGURE 2

Protein loop dynamics impact drug binding kinetics. (a) Ordering of a loop controls the slow-onset inhibition of *Mycobacterium tuberculosis* enoyl-ACP reductase (InhA) by PT70 [14]. Crystal structures of InhA bound to NAD⁺ only (left) or NAD⁺ and the inhibitor PT70 (yellow; right). Rational modification of a fast-on, fast-off triclosan-like InhA inhibitor, by addition of a single methyl group, gave the slow-on, slow-off inhibitor PT70. Binding of PT70 causes the initially disordered loop (red; left; not resolved in the crystal structure) to adopt an ordered helical conformation (red; right). This ordering is thought to be the cause of the slow-onset and slow-off inhibition associated with this and similar inhibitors. (b) Conformational dynamics of an extracellular loop could explain the kinetic selectivity of tiotropium for the M3 muscarinic receptor over the M2 receptor [15]. The conformation of extracellular loop 2 (ECL2) is virtually identical in M2 and M3 crystal structures, but simulations show that M3 ECL2 is more rigid than M2 ECL2. A snapshot from a molecular dynamics simulation (green) of M2 superimposed onto the crystal structure (gray) viewed from the extracellular side into the binding pocket. ECL2 and the beginning of helix 5 (red) undergo a large, reversible conformational fluctuation in M2 that creates a more open pathway for tiotropium escape; similar simulations with M3 reveal no such flexibility. This extra rigidity of M3 could explain the 10-fold longer residence time of tiotropium in M3 despite its similar affinity for both receptors.

Electrostatics

Electrostatic interactions between a charged drug and a charged receptor impact association and dissociation rates, similarly to the effects electrostatics has upon protein–protein binding. Altering the solution ionic strength can greatly affect association rates: increasing ionic strength decreases on-rates but hardly affects off-rates (cf. Debye–Hückel theory [20,21]). Other than as a test for the importance of electrostatics in modulating binding kinetics, however, the pharmacological relevance of this common laboratory manipulation is unclear, because physiological ionic strength is relatively constant. On-rates can also be very sensitive to long-range electrostatic attraction (or repulsion). Off-rates can be modulated by electrostatics, but they tend to be influenced more by short-range drug–receptor interactions such as hydrogen bonds, salt bridges and van der Waals (especially hydrophobic) contacts [22,23]. Binding of a charged acetylcholinesterase inhibitor, for instance, was ~50-fold faster, and unbinding ~10-fold slower, than that of a nearly identical neutral analog in which the inhibitor's trimethylammonium group was changed, by one atom (N⁺ to C), to the *t*-butyl isostere [24].

Experimental studies have shown that it can be difficult to distinguish the electrostatic effects on binding kinetics from the effects of other molecular determinants; deviations from the simple picture described above are not uncommon. The effective charge of a drug or receptor does not necessarily equal its formal charge, and, paradoxically, ‘charge matching’ (i.e. negative paired with positive) is not necessarily required for rapid binding [25]. The former issue is demonstrated, for instance, by the insensitivity of the binding rates of nucleotide di- or tri-phosphates to the Na⁺/K⁺-ATPase, despite the

ligand charge varying from –0.8 to –3.8 [23]. Binding of phosphate to (negatively charged) periplasmic phosphate binding protein illustrates the latter issue: surprisingly, despite ‘mismatched’ charges, the association rate is nearly diffusion controlled [26]. In a similar vein, increasing charge complementarity has been observed to *decrease* association rates in certain cases [25], and large alterations in (receptor) charge lead to only minor changes in the on-rates of carbonic anhydrase inhibitors [27].

Hydrophobicity and water

Although the influence of water on the stabilization of drug–receptor complexes is well known (the hydrophobic effect) [28], the effect of water on binding kinetics has only recently been recognized [29–36]. At small length scales, on the order of several ångströms, the motion of a few water molecules can be enough to influence binding kinetics. Using a combination of experiment and computer simulations, Schmidtko and co-workers showed that, when a ligand and a receptor interact via hydrogen bonds shielded from water by surrounding hydrophobic regions, the resulting complex tends to be more kinetically stable than if the hydrogen bonds were less shielded [36] (Fig. 3). The difficulty with which water diffuses into and away from these largely hydrophobic sites appears to create a kinetic barrier to ligand binding and unbinding.

At larger length scales involving nanometer-scale volumes of water, collective water motion out of a hydrophobic region, or ‘dewetting’, can present a barrier to drug entry [32,35,37]. Recently, Setny and co-workers explicitly demonstrated the existence of a dewetting barrier to ligand binding in computer simulations of a model system [32]. As the ligand approached the receptor

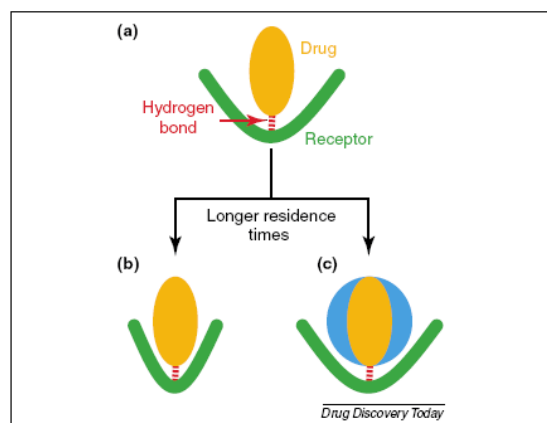


FIGURE 3

Shielded hydrogen bonds confer longer residence time. (a) Schematic of a drug (yellow) bound to a receptor (green), forming, among other interactions, a hydrogen bond (red dashed line). (b) Compared with (a) the greater curvature of the binding site shields the hydrogen bond from water access, creating a larger barrier to drug dissociation. (c) For a less-curved binding site, as in (a), increasing the ligand size (blue) also shields the hydrogen bond. The difficulty with which water diffuses into and away from a shielded hydrogen bond directly impacts drug residence time, by creating a kinetic barrier to ligand binding and unbinding [36].

a barrier arose between a wet and dry binding pocket. Surmounting this dewetting barrier presented the major bottleneck to ligand entry. In simulations of beta blockers binding to β -adrenergic receptors we observed a qualitatively similar phenomenon where entry of the hydrophobic ligand into a hydrophobic extracellular vestibule was correlated with the collective evacuation of water from that site and from around the ligand [35]. This dehydration step corresponded to the largest energetic barrier along the drug binding pathway.

Methods to quantify molecular determinants

Although techniques for measuring the rates of ligand binding have existed for decades [9,38], methods for correlating such kinetic data with molecular determinants have only emerged recently. These newer techniques provide insight into how kinetics is influenced by drug and receptor structure.

A traditional approach to probing the molecular determinants of binding kinetics is to combine site-directed mutagenesis with a rate-measurement technique, such as surface plasmon resonance [39] or radioligand binding [40]. Observing how different protein mutations affect rates provides an indirect way of identifying those molecular features that impact binding kinetics. Zhukov and co-workers at Heptares Therapeutics have, for instance, applied this idea in a systematic manner by measuring k_{on} , k_{off} and K_d for a large number of receptor mutants and small-molecule ligands [41].

Another recent approach uses NMR relaxation dispersion experiments to probe protein dynamics during ligand binding and unbinding. Carroll and co-workers investigated the binding of a series of 2,4-diaminopyrimidine inhibitors to dihydrofolate reductase; they found that inhibitor dissociation rates are correlated with

the rate of a protein conformational switch located near the active site [42].

All of these methods, however, only provide indirect evidence about transient structures visited along a ligand-binding pathway. Direct, atomic-resolution structural information, especially for the binding transition state, would provide the best basis for the rational modulation of binding kinetics. One experimental method that has been used to characterize transition states is the measurement of kinetic isotope effects, typically for enzymatic reactions [43]. It is conceivable that this method could provide detailed information on the nature of binding transition states useful for rational optimization of binding kinetics. Another method under active development, which might be able to obtain similar information on fleeting intermediates or transition states, is time-resolved Laue X-ray crystallography [44].

By their very nature, computational methods, in particular molecular dynamics simulations, provide detailed structural information on metastable intermediate states and transition states, at atomic spatial and femtosecond temporal resolution [45]. Owing to increases in computational power, it has recently become possible to simulate the full process of spontaneous ligand–receptor association — which typically occurs on the microsecond timescale — in atomic detail, providing direct access to detailed information on binding mechanisms that have been difficult to access experimentally [31,35,46,47]. In recent work from our group, molecular dynamics simulations of the spontaneous binding of several drug molecules to kinases and GPCRs achieved bound poses virtually identical to the crystallographically determined bound structures. Estimates of on-rates from simulation were also in approximate agreement with experimental measurements [35,47]. Although the physicochemical models underlying molecular dynamics simulations remain imperfect, these and other studies demonstrate the beneficial use of such simulations in probing drug binding pathways.

Various other computational methods, ranging from coarse-grained molecular dynamics simulations [48–50] to biased enhanced-sampling simulations [15,51–55], have also been used to characterize binding pathways. Because ligand dissociation is slower — often taking seconds to hours — it can usually be observed computationally only by use of these latter techniques. It is important to note, however, that in the absence of external driving forces the unbinding process is the reverse of the binding process, following the same pathway and traversing the same barriers in the opposite order.

Concluding remarks

Our current understanding of the factors influencing binding rates remains incomplete. The future design of drugs that possess specific receptor–interaction kinetics will ideally involve detailed characterization of not only the bound state but also the entire drug–receptor binding pathway, including metastable intermediate states and transition states. With such a complete understanding will come the insights needed to guide the modification of particular molecular features to affect binding rates in the desired manner. In the meantime, we conclude by discussing briefly how the principles we have reviewed could be used today.

How might drug residence time be modulated in a rational manner? One obvious brute-force approach to increase, for

example, drug residence time is simply to make larger molecules. Unfortunately, the significant disadvantages of increased drug size, well known from decades of binding-affinity optimization, limit the benefit of such an approach to optimizing kinetics. Increasing the rigidity of a drug candidate is a related approach that can increase residence time without adding too much bulk. In some cases, however, flexible groups can enable the drug to access slowly dissociating bound conformations, as exhibited by the long, branched aliphatic substituents of the aforementioned corticotropin-releasing factor receptor antagonists [11]. Should shorter residence times be desirable, smaller, more-flexible molecules might be needed. However, operating in such a regime demonstrates the difficulty of separately modulating k_{on} , k_{off} and K_d (Box 1).

A more nuanced route to modulating residence times could involve changing interactions between the drug and those fluctuating parts of the receptor that often appear to be the bottlenecks to drug binding. In these cases, small, rational changes to the drug — for instance adding a group to make a specific interaction, as in the extra methyl group of the InH_A inhibitor PT70 [14], or removing a group to break a specific interaction — could result in large changes in residence time. Notably, the atomic groups modified in this way need not be those that confer (the bulk of) binding affinity; in the same way that solubilizing groups can often be added, in an almost orthogonal manner, to a drug binding-core template. Such a design strategy is also promising from the viewpoint of subtype selectivity, especially for receptors in which the binding site is well conserved among subtypes. Because the energetic barriers that determine binding kinetics often arise in non-conserved regions, distal from the binding site, it might prove easier to design drugs with residence times that differ among receptor subtypes than to design drugs with differing affinities.

Harnessing electrostatic interactions to optimize binding kinetics might require a similarly subtle approach, because substantial modifications to the charge of a drug often cause significant and often irrevocably deleterious changes in ADME properties. One such approach is the modulation of amine basicity, which changes the proportion of drug molecules that are charged at physiological pH; this approach is already used to alter affinity (e.g. piperidine versus morpholine). The acidity of other functional groups (e.g. carboxylates, *N*-aryl-sulfonamides [27], etc.) can be manipulated in a similar manner. We believe this tactic could thus represent a viable approach to rational optimization of binding kinetics. A more subtle approach would be to alter the distribution of charges in the drug, thereby modulating particular interactions (e.g. the strength of a hydrogen bond that contributes to k_{off}) more than others.

Residence time can also be modulated by leveraging water dynamics. Increasing the number of shielded hydrogen bonds, or accentuating the hydrophobic shielding of existing hydrogen bonds by designing a broader ligand, could tend to increase residence time (Fig. 3). At larger length scales where the driving force of drug binding is controlled by dewetting, adding hydrophobic groups to the ligand might lower the dewetting barrier [32,37].

With experimental methods for determining drug binding kinetics becoming faster and less expensive, the availability of such data will surely become more widespread and the drive to incorporate it into drug discovery programs will increase. A greater understanding of the molecular determinants of binding kinetics will be crucial for maximizing the impact kinetics data has on drug discovery.

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