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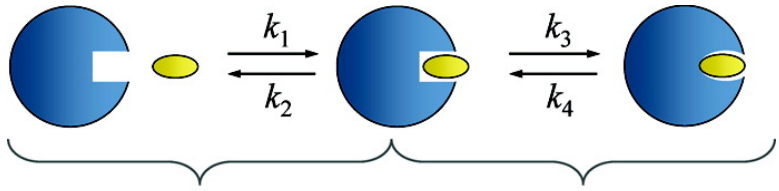

$$K_d = \frac{k_2}{k_1} \qquad K_d^* = \frac{K_d}{1 + \left(\frac{k_3}{k_4}\right)}$$

$$k_{off} = \frac{k_2 k_4}{k_2 + k_3 + k_4} \qquad \tau = \frac{1}{k_{off}} \qquad t_{1/2} = \frac{0.693}{k_{off}}$$

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Current Topics

Residence Time of Receptor−Ligand Complexes and Its Effect on Biological Function

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ABSTRACT: The formation and duration of binary receptor−ligand complexes are fundamental to many physiologic processes. Most often, the effectiveness of interaction between a receptor and its ligand is quantified in terms of closed system, equilibrium affinity measurements, such as IC_{50} and K_d. In the context of *in vivo* biology, however, the extent and duration of responses to receptor−ligand interactions depend greatly on the time period over which the ligand is in residence on its receptor. Here we define receptor−ligand complex residence time in quantitative terms and describe its significance to biological function. Examples of the importance of residence time are presented for natural ligands of different receptor types. The impact of residence time on the optimization of potential ligands as drugs for human medicine is also described.

Essentially all of the biochemical activities of cell physiology are mediated by the transient formation of binary complexes between macromolecular receptors and their ligands. Thus, for example, enzymatic activity depends on substrate and sometimes allosteric activator binding to enzymes, signal transduction depends on agonist engagement by cell-surface, G-protein-coupled receptors, gene transcription depends on binding of transcriptional activators to specific promoter regions of the gene, and cell cycle progression and mitosis depend on a myriad of receptor−ligand interactions. For the vast majority of these biological activities, the binary complex between receptor and ligand has a finite lifetime, so the functional consequences of complex formation are likewise transient and can be tightly regulated in terms of timing of initiation, duration of action, and amplitude of action. Much of the critical regulation of biological activity within a cell is also mediated by receptor−ligand interactions, often in the form of binding interactions between a receptor and an inhibitory ligand. When these systems are dysregulated, the consequences can be devastating to the cell and/or organism. When, for example, cell cycle progression and proliferation are dysregulated, hyperproliferation occurs, leading to diseases such as cancer (*1, 2*).

Additional cellular regulation mechanisms, such as apoptosis (*3*) and autophagy (*4*), are also in place to prevent unregulated cell division, and these too depend on well-orchestrated receptor−ligand interactions that can be dysregulated in disease states. Attempts to intervene pharmacologically in these diseases often involve the administration of low-molecular weight xenobiotics (i.e., drugs) that themselves function by receptor binding, effecting activation

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(agonists) or inhibition (antagonists) of specific, disease-associated receptors.

In most, but not all, physiological situations, the duration of the biological effect produced by a receptor−ligand complex is directly related to the lifetime of the binary complex itself. Thus, the longer the ligand is in residence at its receptor, the longer the biological effect endures, especially when that effect is inhibition of biochemical function. For example, residence times for substrates bound to an enzyme are relatively short, averaging 50 ms.[1] On the other hand, naturally occurring, reversible inhibitors of enzymes tend to have a much longer residence time, as the long residence time translates into sustained inhibition of enzymatic activity. Basic pancreatic trypsin inhibitor, for example, inhibits trypsin with a receptor−ligand residence time of 1.52×10^7 s, or approximately 6 months.[2] Likewise, many small molecule drugs that act as effective, targeted, enzyme inhibitors and receptor antagonists display residence times in the range of hours to days (*vide infra*).

Most commonly, the effectiveness of interaction between a receptor and ligand is quantitatively assessed by equilibrium measures of binding affinity, such as IC_{50}, the equilibrium dissociation constant, or the Gibbs free energy of binding. These thermodynamic constants are related to the kinetic rate constants for complex association and dissociation in different ways, depending on the mechanism of complex formation (*vide infra*). In many cases, one finds that complex association rates for different ligands, binding to a common receptor, are very similar. Therefore, differences in complex dissociation rates among the ligands translate directly into differences in the thermodynamic constants. In such situations, one could conclude that measurement of the equilibrium thermodynamic constant is sufficient for defining differences in complex lifetimes. However, in our experience, exclusive reliance on such equilibrium measurements can be misleading with respect to relative complex lifetime. Even within a common chemical series of ligands, where association rates generally do not vary significantly, we have encountered cases in which two ligands displayed similar K_d values but vastly different dissociation rates.

As just described, the affinity of a ligand for its receptor does not, *per se*, define the effectiveness and duration of biological action. Rather, it is the lifetime of the binary receptor−ligand complex that in large part dictates the effect in the cellular and organismal context. Recently, Copeland et al. (*5*) and independently Vauquelin et al. (*6, 7*) and Swinney (*8*) have discussed this concept with regard to the pharmacological activity of drugs. Copeland et al. (*5*) defined the term residence time to refer to the temporal duration of the binary receptor−ligand complex. These authors argued that while the lifetime of a receptor−ligand complex is affected both by the rate of ligand association with and dissociation from the receptor, the key driver of complex lifetime *in vivo* is, in fact, the dissociation rate. The term residence time (τ), defined as the reciprocal of the dissociation rate constant (k_{off}) for the receptor−ligand complex, was coined as an experimentally measurable, quantitative representation of complex lifetime, or duration. To illustrate the concept, consider how a violinist can control the duration of a musical note by the length of time that the bow is drawn across a specific string (how rapidly the violinist initially places the bow on the string determines the timing of when the note begins but does not affect its temporal duration). A quick "pluck" at the string results in a note of very short duration. A slow draw of the entire length of the bow across the string produces a prolonged note. Thus, the duration of the note (analogous to biological function) is directly related to the residence time of the bow (analogous to ligand) on the violin string (analogous to the receptor).

In this work, we discuss the concept of residence time in the broader context of the biological activities of receptor−ligand complexes in general. We will review the key concepts associated with receptor−ligand complex residence time and describe examples of the relationship between receptor−ligand residence time and cellular and organismal biology. We also describe the application of residence time measurements to drug optimization efforts in the field of human medicine.

CLOSED AND OPEN SYSTEMS IN BIOLOGY

Before considering the effects of residence time on receptor−ligand complex activity, it is useful to first identify two common sets of conditions under which receptors and ligands encounter one another. The first is termed a closed system. Here the total receptor and ligand concentrations are constant, meaning that the total concentrations are both homogeneous and unchanged with time. These conditions are representative of most experimental measurements of receptor−ligand binding (see refs *5*, *9*, and *10* for common, experimental measures of receptor−ligand binding kinetics and off-rate determinations under closed system conditions). In a closed system, the only change in concentration that takes place with time is in the concentrations of free and bound species as the system approaches equilibrium. In most instances, binding measurements are performed after sufficient mixing time to ensure that the system has reached equilibrium, to ensure that measurements of thermodynamic constants can be performed accurately. As discussed elsewhere (*5, 10*), in favorable cases the kinetics of approach to equilibrium in a closed system experiment can be used to determine the rate constants for complex association and dissociation.

The second type of system is termed an open system. This is more reflective of how receptors and ligands encounter one another *in vivo*. Here one of the components (typically the receptor) is held within a defined container (e.g., a cell) at a fixed concentration. The other component (most commonly, the ligand) flows into the container harboring the receptor, diffuses within the container to encounter the receptor, and may also flow out of the container by passive and/or active transport mechanisms. For example, when a drug that targets an intracellular receptor is administered orally, it must enter the gastrointestinal tract, be absorbed from the intestines into systemic circulation, be thus transported to the tissue of interest, diffuse through the tissue to reach the cell, be transported into the cell, and then diffuse to the receptor before association (binding) can occur. Once bound, the ligand can dissociate from the receptor and may rapidly rebind, due to the high local concentration of ligand in the proximity of the receptor. Alternatively, the dissociated

[1] Calculated from tabulated values of enzyme substrate off rates in ref *63*.

[2] Calculated from the off rate value in ref *63*.

ligand may diffuse through the cytosol, or other subcellular compartments, and eventually be transported back out of the cell. Within and outside the cell, various chemical (e.g., digestion) and metabolic processes may degrade the ligand to eliminate it from the body. Hence, in contrast to the constant concentration encountered in a closed system, the open system is characterized by continuous changes in the flux of ligand available for encounter with the receptor. Because the free concentration of ligand is continuously in flux in an open system, equilibrium measurements are no longer appropriate. Measurement of the rate constants for binary complex association may be possible but in many cases is compromised by other processes that limit access of the ligand to the receptor (*5*). On the other hand, the dissociation rate for the binary complex is first-order and thus independent of any changes in free ligand concentration.

EQUILIBRIUM AND KINETIC ASPECTS OF RECEPTOR−LIGAND INTERACTIONS

Most biochemists are familiar with equilibrium measures of receptor−ligand binding. The strength of interaction between a ligand and its receptor is typically defined in terms of thermodynamic constants, measured *in vitro* under closed system, equilibrium conditions, such at the Gibbs free energy of binding ($\Delta G_{binding}$), the equilibrium association constant (K_a), and most commonly the equilibrium dissociation constant (K_d). These constants are quantitatively related to one another by eq 1.

$$\Delta G_{binding} = -RT\ln(K_a) = RT\ln(K_d) \quad (1)$$

where R (1.987 cal °C^{-1} mol^{-1} or 8.314 J °C^{-1} mol^{-1}) is the ideal gas constant and T is the temperature in Kelvin.

These thermodynamic constants serve well to define complex affinity under equilibrium conditions for both open and closed systems. As described above, and elsewhere (*5, 6, 10*), however, equilibrium complex affinity, per se, is not the key driver of durable biological effect in open systems. Rather, the overall rate of dissociation of the receptor−ligand complex is proposed to be the most critical component of complex lifetime and, hence, biological function, *in vivo*.

The overall rate of binary complex dissociation is termed the off-rate and is represented by the symbol k_{off}. The definition of this constant, however, in terms of individual, microscopic rate constants, depends on the specific mechanism of receptor−ligand interaction. There are three general mechanisms of interaction that one may envisage for a receptor−ligand pair. The first (mechanism A) is a simple one-step binding and one-step dissociation process.

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL$$

Here the receptor (R) and ligand (L) combine to form the binary complex (RL) with the association rate constant (k_{on}) equal to k_1 and k_{off} equal to k_2. The equilibrium dissociation constant (K_d) associated with this mechanism is simply given by the ratio k_2/k_1. One can measure the kinetics of the approach to equilibrium in a system like this by a number of experimental measures (*9, 10*). If either k_2 or both k_1 and k_2 are slow on the time scale of the experimental measurements, the concentration of RL over time appears to follow a pseudo-first-order process described by the observed pseudo-first-order rate constant k_{obs}. A plot of k_{obs} as a function of ligand concentration is linear (Figure 1A), and this is a distinguishing feature of mechanism A. The off-rate (k_{off}) for this mechanism is equivalent to the microscopic rate constant k_2, and the residence time (τ) is given by the reciprocal of k_2. For some researchers (e.g., those in the pharmacology and drug discovery fields), the concept of half-life is more familiar than relaxation or residence time. Hence, Copeland et al. (*5*) have also coined the term dissociative half-life ($t_{1/2}^{diss}$) to describe the half-life of the receptor−ligand complex, which for mechanism A is simply $0.693/k_2$ (for all mechanisms, $\tau = 1/k_{off}$ and $t_{1/2}^{diss} = 0.693/k_{off}$).

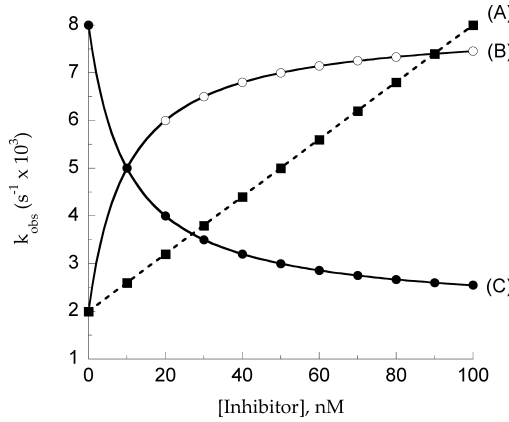

FIGURE 1: Experimentally observed, pseudo-first-order rate constant, k_{obs}, as a function of ligand concentration for different mechanisms of receptor−ligand binding. (A) Single-step binding mechanism termed mechanism A in the text (■). (B) Two-step binding mechanism termed mechanism B in the text (○). (C) Two-step binding mechanism termed mechanism C in the text (●).

The second common mechanism of receptor−ligand interaction (mechanism B) is analogous to the induced-fit model of enzyme−substrate interactions, first postulated by Koshland and co-workers (*11*).

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \underset{k_4}{\overset{k_3}{\rightleftharpoons}} R^*L$$

Here the ligand initially encounters the receptor in a conformational state (R) that is less than optimally complementary to the ligand for binding. Subsequent to binding, the receptor undergoes an isomerization to a new conformational state (R*) that is much more complementary to the ligand such that the new binary complex R*L has a much higher affinity than the initial encounter complex, RL. Thus, this mechanism cannot be described by a single equilibrium dissociation constant; instead, two equilibrium dissociation constants are required here. The affinity of the initial encounter complex RL is quantified by K_d which is still defined by the ratio k_2/k_1. The affinity of the final complex R*L, however, is defined by a more complex equilibrium constant given the symbol K_d* (see Table 1 for the mathematical definition of K_d*). The transition between the initial encounter complex and the final binary complex often imparts a rate limit to the overall approach to the final equilibrium state such that the approach to equilibrium again is described by the experimental rate constant k_{obs}. For mechanism B, a plot of k_{obs} as a function of ligand concentration yields a hyperbolic curve as illustrated in Figure 1B.

The value of k_{off} for mechanism B is not simply defined by a single, microscopic rate constant. Instead, it is a

Table 1: Mathematical Definitions of Equilibrium and Kinetic Constants for Different Mechanisms of Receptor−Ligand Interactions

Kinetic Parameter	Mechanism A[a]	Mechanism B[a]	Mechanism C[a]
K_d [b]	$\frac{k_2}{k_1}$	$\frac{k_2}{k_1}$	not applicable
K_d* [c]	not applicable	$\frac{k_2}{\left(k_1+\frac{k_1k_3}{k_4}\right)}=\frac{K_d}{\left(1+\frac{k_3}{k_4}\right)}$	$\frac{k_4}{k_3}$
k_{obs} [d]	$k_1[L]+k_2$	$k_4+\frac{k_3k_1[L]}{k_2}=k_4+\frac{k_3}{1+\frac{K_d}{[L]}}$	$k_1+\frac{k_2}{\left(1+\frac{k_3[L]}{k_4}\right)}=k_1+\frac{k_2}{\left(1+\frac{[L]}{K_d^*}\right)}$
k_{off} [e]	k_2	$\frac{k_2k_4}{(k_2+k_3+k_4)}$	k_4
τ [f]	$\frac{1}{k_2}$	$\frac{(k_2+k_3+k_4)}{k_2k_4}$	$\frac{1}{k_4}$
$t_{1/2}^{diss.}$ [g]	$\frac{0.693}{k_2}$	$\frac{0.693(k_2+k_3+k_4)}{k_2k_4}$	$\frac{0.693}{k_4}$

[a] Mechanisms in this table refer to the three mechanisms of receptor−ligand interaction discussed in the text. [b] K_d is the equilibrium dissociation constant for the initial encounter complex RL. [c] K_d* is the equilibrium dissociation constant for the final binary complex R*L in a two-step binding mechanism. [d] k_{obs} is the experimentally observed pseudo-first-order rate constant for the approach to equilibrium between the free components and the binary receptor−ligand complex. [e] k_{off} is the overall rate of dissociation of the receptor−ligand complex. [f] τ is the residence time of the receptor−ligand complex. [g] $t_{1/2}^{diss}$ is the dissociative half-life of the receptor−ligand complex.

compilation of several rate constants associated with the initial encounter complex and the forward and reverse isomerization steps of RL to R*L conversion (see Table 1). In most instances of long residence time for this mechanism, it is the reverse isomerization rate constant, k_4, which is slowest and hence limiting with respect to complex dissociation. However, it can also be the case that k_2 and k_4 are similar in magnitude and thus both are limiting to complex dissociation (see ref *12* for an example of this situation). In either case, the receptor isomerization step adds significant potential for a long-lived binary complex between the receptor and its ligand. Mechanism B is very commonly encountered in situations of high-affinity receptor−ligand interactions, as is often the case, for example, for pharmacologically active enzyme inhibitors and receptor antagonists (*10*).

A third mechanism for receptor−ligand interactions is represented by mechanism C.

$$R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R^* \underset{k_4}{\overset{k_3[L]}{\rightleftharpoons}} R^*L$$

In this mechanism, the receptor is in equilibrium between two conformational states in the absence of ligand. One of these conformational states is competent to bind ligand (R*), and the other is not (R). The introduction of ligand into the system captures the subpopulation of receptor in the R* state and thereby depletes the pool of free R*. In response, the equilibrium is shifted in favor of formation of R* from the remaining pool of free receptor molecules. With a sufficient ligand concentration and time, the entire population of receptor can be driven into the binary R*L complex state. For this mechanism, the interconversion between conformational states R and R* is slow relative to the binding of ligand to the R* state. Again, the overall approach to equilibrium for this mechanism gives the appearance of pseudo-first-order kinetics. In stark contrast to the other two mechanisms, for mechanism C the value of k_{obs} actually *decreases* with increasing ligand concentration, asymptotically approaching a finite value at very high ligand concentrations (Figure 1C). This is an extremely clear, distinguishing feature of mechanism C that allows unambiguous differentiation from mechanisms A and B. We include mechanism C here for completeness but feel compelled to point out that this mechanism is rarely encountered in receptor−ligand binding studies. In fact, we know of only two examples of mechanism C in the biochemical literature. One example comes from studies of *Escherichia coli* alkaline phosphatase binding and hydrolysis of the substrate 2,4-dinitrophenyl phosphate (*13*), and the second comes from studies of chymotrypsin binding and hydrolysis of aromatic substrates (*14*). Recent crystallographic studies of several kinases bound to high-affinity, ATP-competitive inhibitors have revealed that the protein adopts a conformation in the inhibitor-bound form that resembles that of the inactive state of the kinase. This "inactive state" is typically accessed only prior to an activating phosphorylation of the target kinase by another kinase upstream of it in an intracellular signal transduction pathway. This has led some researchers to invoke mechanism C and suggest that the inhibitors bind to an inactive state subpopulation of kinase molecules in solution that is in

equilibrium with the activated state of the kinase. To our knowledge, however, there is a paucity of kinetic data that would definitively distinguish this proposed mechanism of kinase inhibition from that represented by mechanism B. Thus, further experimental work is needed to make clear this mechanistic distinction, as it has potentially important implications for how one conducts future drug-seeking efforts against such kinases.

The mathematical definitions for equilibrium and kinetic constants associated with mechanisms A−C are summarized in Table 1.

EXAMPLES OF RESIDENCE TIME IN NATURAL RECEPTOR−LIGAND FUNCTION

Open biological systems, as defined above, create high hurdles for the specificity and stability required of receptor−ligand mediated biological functions. Ligand concentrations at the receptor site change over time, and in some cases, the ligand is not colocalized with the receptor at the cellular or tissue level. Additionally, some multistep biosynthetic functions have a strict requirement for fidelity, and open systems increase the already high entropic barrier that exists in these cases (see below). In many cases, biological systems employ the receptor−ligand residence time, varying across a wide range, as one method of overcoming these hurdles. A comprehensive discussion of the myriad of physiological examples of such controlled biological response is not possible in this brief review. Rather, we exemplify these concepts with descriptions of selected classes of receptor−ligand complexes for which residence time is a clear and important component of biological function.

Immune Response. Adaptive immune response involves the formation of binary complexes between ligands and receptors on B and T lymphocytes. Antigen receptors, composed of immunoglobulin domains, on the surface of B-cells recognize and bind specific antigens such as proteins from pathogenic bacteria and viruses. The receptor−antigen complex on the B-cell surface undergoes endocytosis and intracellular proteolytic processing to produce antigen-derived peptides. These peptides are then presented on the surface of the B-cell in complex with a major histocompatibility complex (MHC) class II protein. The peptide−MHC complex (pMHC) then acts as a ligand for the T-cell receptor (TCR), in concert with the CD4 coreceptor, on the surface of T-helper cells. Engagement of TCR/CD4 by the pMHC leads to T-helper cell activation, resulting in cytokine secretion that stimulates a number of cellular responses, including B-cell proliferation and, hence, antigen specific antibody production. In a like manner, the TCR on killer T-cell, together with its coreceptor CD8, engages antigen−MHC class I complexes on cell surfaces, stimulating secretion of the cytotoxin by the killer T-cell. These cytotoxins target the plasma membrane of the antigen-presenting cell, resulting in apoptotic cell death.

Several studies have explored the kinetics of interactions between various components of the adaptive immune system. Among a large number of studies of soluble antibody−antigen interactions, some common features emerge (*15*). Often association of the antibody with the antigen is slower than expected for a diffusion-controlled process, and this has been suggested to result from a two-step, induced-fit mechanism of binding (mechanism B, above). Dissociation of antibody−antigen complexes is generally found to be slow, with residence times ranging from minutes to days or longer. For example, Katsamba et al. (*16*) studied the binding of prostate specific antigen (PSA) with several antibodies in a number of independent laboratories. The average value of k_{on} was 4.1×10^4 M^{-1} s^{-1}, and the values of k_{off} translated into an average residence time of 6.2 h for these complexes. Several studies comparing on-rate, off-rate, and biological function among various antibodies for a common antigen have shown that optimal function is often correlated with off-rate or residence time. For example, VanCott et al. (*17*) looked at a panel of human and mouse antibodies specific for the V3 loop of the HIV-1 envelope glycoprotein gp120. These workers found that the variation in k_{on} across the antibody panel was only 4-fold, while the values for k_{off} varied more than 100-fold. The concentration-dependent ability of the antibodies to neutralize viral replication was also assessed by measuring the concentration of antibody required to inhibit cellular p24 production by 50% (N_{50} value). A plot of k_{off} and N_{50} showed a significant correlation between these values ($R^2 = 0.75$; $p < 0.001$), while a similar plot of k_{on} and N_{50} demonstrated no correlation ($R^2 = 0.002$; $p = 0.92$). This is typical of the results seen for other antibody−antigen complexes, with k_{on} values ranging from 10^4 to 10^7 M^{-1} s^{-1} and residence times ranging from a few seconds to several months (*15*).

Similarly, the kinetics of interaction between antigens and B-cell surface receptors have been studied by several groups. With respect to recognition specificity, Foote and Eisen (*18*) point out that the most rapid association between antigens and B-cell receptors is limited by the diffusion coefficients of the reactants, which puts an upper limit on k_{on} of ca. 10^6 M^{-1} s^{-1} for monomeric protein antigens. Since structural complementarity has no impact of diffusion-controlled processes, the encounter rate cannot be a factor in discrimination among antigens. As with soluble antibodies, however, there are experimental data to suggest that antigen binding to B-cell receptors occurs through a two-step, induced-fit process [see the work of Sundberg and Mariuzza (*19*) for a summary of these data]. Once an antigen is bound at the B-cell receptor, the complex must be internalized through endocytosis to elicit a downstream immune response. If the antigen dissociates from the receptor prior to endocytosis, complex formation cannot elicit a functional response. Hence, immune response requires some minimum residence time for the antigen−receptor complex (*20*). On the other hand, a residence time for the antigen−receptor complex that is much longer than the time required for endocytosis does not provide a further functional advantage. Foote and Eisen (*18*) estimated the half-life for endocytosis to be 8.5 min. They assumed that two to three half-lives define an upper limit beyond which increased complex stability offers no additional advantage. Hence, the effective limit of B-cell receptor−antigen residence time can be estimated to be between 0.5 and 3 h. Within the limits of minimal and maximal effective residence time and affinity, Guermonprez et al. (*20*) present data to suggest that the efficiency of B-cell-mediated presentation of antigenic peptides to T-cells is controlled by the off-rate of the antigen−receptor complex, with slower off-rates correlated with an increased level of signaling. Thus, complex stability, dictated by residence time,

is a critical factor for ensuring that the binding of antigens to the B-cell receptor translates into functional processing of the antigen.

Once antigen is proteolytically processed within the B-cell, the resulting peptides must form stable, noncovalent complexes with MHC proteins on the surface of the B-cell, to facilitate recognition by the TCR and subsequent T-cell activation. Again, the stability of the pMHC binary complex is critical to further immune response, and this is dictated by the residence time of the complex. The pMHC binary complex exists in an open system in serum with the TCR on T-cells. A long residence time for the pMHC binary complex is important in overcoming the entropic barrier to formation of the pMHC complex−TCR ternary complex. Margulies et al. (*21*) studied the kinetics of interactions of MHC class I with antigen-derived peptides and also summarized previous studies of interactions of MCH class II with peptides. For both MHC classes, the residence times of the pMHC binary complexes ranged from 3 h to 12 days. These long residence times ensure that the peptide−MHC binary complex endures for sufficient time to affect TCR engagement.

Binding of the pMHC to the TCR also appears to proceed through a two-step, induced-fit mechanism, characterized by slow association kinetics, large heat capacity changes, and a large entropic barrier to binding (*19*). These results are bolstered by comparisons of the crystallographic structures of unliganded and pMHC-bound TCRs, which also suggest significant conformational adjustments attending pMHC binding. Boniface et al. (*22*), for example, found that the large heat capacity changes that attend pMHC binding to TCR, and a large contribution of conformational entropy to the overall entropic barrier to binding, could not be ascribed to rigid-body association between the binding partners. Rather, the data are indicative of an induced-fit mechanism of binding. Boniface et al. (*22*) go on to suggest that for pMHC−TCR interactions, like DNA−protein interactions, recognition specificity is not due to rigid alignment of preexisting, complementary surfaces but instead requires unstructured regions of the protein(s) in the free state to fold upon binding to create key parts of the recognition contact interface.

In general, the residence time of the pMHC−TCR complex directly correlates with the degree of T-cell activation. However, Krogsgaard et al. (*23*) noted that there are exceptions to this generality. In a detailed study of binding kinetics and thermodynamics, these workers demonstrated for a single TCR interacting with a series of pMHCs that the degree of T-cell activation could be accurately predicted by taking into account both the half-life of the pMHC−TCR complex and the change in heat capacity upon binding. These data suggest that the combination of induced fit and the consequent longer residence time of the complex is a critical factor for the specificity and effectiveness of interactions of pMHC with the TCR.

Control of Proteinase Activity by Natural Inhibitors. Proteinase-catalyzed hydrolysis of specific peptide bonds in proteins and peptides is essential for a broad range of physiological functions, including digestion, immune response, xenobiotic metabolism, blood clotting, wound healing, and tissue remodeling. Intracellular proteolysis, due to the action of proteases, plays important roles in processes such as signal transduction, exocytosis, endocytosis, and apoptosis. As essential as these activities are to life, the uncontrolled activity of proteolytic enzymes would be devastating to the organism. Hence, nature has developed several strategies to ensure that these enzymes remain inactive until needed for specific cellular or extracellular functions. A common strategy for inactivating proteinase in nature is to form high-affinity, long-lasting binary complexes between the proteinase and a protein-based inhibitor. Indeed, the residence times for binary complexes between proteinases and protein-based inhibitors are among some of the longest found in nature, reflecting the need for tight control of proteinase activity *in vivo*. Janin and Chothia (*15*) surveyed the structures and binding kinetics for a representative set of 15 pairs of proteinase−inhibitor complexes. Generally, the contact interface for the complex consisted of a long groove formed by the active site and specificity pockets of the proteinase and an extended loop of the inhibitor that fits into the proteinase groove. The contact areas for the complexes range from 600 to 1000 Å^2 and are dominated by nonpolar residues. Among these 15 proteinase−inhibitor complexes, Janin and Chothia found that there was little variation in the association rate constants and concluded that affinity changes and inhibitor specificity were instead dicated by the dissociation rate constants (k_{off}). The residence times among these complexes range from 1 s for low-affinity complexes to 4 months for the most stable complexes. Further analysis of the kinetic data from the literature led Janin and Chothia to conclude that association was likely to result from a loose encounter complex that isomerizes to a stable structure with proper packing interactions at the protein interface. They noted that experimental evidence for two-step binding mechanisms had been reported from kinetic studies of a number of proteinase−inhibitor pairs.

One of the best-studied examples of a natural protein-based proteinase inhibitor is pancreatic trypsin inhibitor (PTI), an inhibitor of trypsin and related serine proteinases. In a detailed study of binding kinetics, Vincent and Lazdunski (*24*) measured the association and dissociation rate constants for trypsin with PTI and for derivatives of each protein. PTI was studied in its natural form and also after selective reduction of the intramolecular disulfide bond between S14 and S38, after reduction and carboxymethylation, and after reduction and carboxamidomethylation. These forms were complexed with natural trypsin, with trypsin that had been selectively reduced and carboxymethylated at S179−S203, and with a catalytically inactivated form of trypsin termed pseudotrypsin. Among these pairs of proteinase−inhibitor combinations, the association rate constants varied only 55-fold, from 2×10^4 to 1.1×10^6 M^{-1} s^{-1}. In contrast, the values of k_{off} spanned a range of 10^5-fold, corresponding to residence times from 22 min to 6 months. Thus, overall complex lifetime is determined almost exclusively by residence time for proteinase−inhibitor complexes, such as the trypsin−PTI complex. Likewise, residence time appears to be an important factor in enzyme−inhibitor specificity for these systems as well.

A final example of the impact of residence time on proteinase−inhibitor stability and specificity comes from studies of tissue inhibitors of metalloproteinases (TIMPs) interacting with various soluble and membrane-bound metalloproteinases. There are four TIMPs (TIMP-1−TIMP-4) that occur naturally and are responsible for controlling the

activity of a range of metalloproteinases. TIMPs were first isolated as complexes with soluble metalloproteinase such as the gelatinases matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9, respectively). Subsequently, an accumulation of data has suggested that the TIMPs may be unique regulators of type I transmembrane metalloproteinases (MT-MMPs) and that this is their key physiological role. Several studies of the kinetics of interaction between TIMPS and MMPs and MT-MMPs quantitatively support this suggestion. For example, TIMP-2 binds to MMP-2 and MMP-9 with K_i values of 7.2 and 43.4 nM, respectively (*25*). In contrast, TIMP-2 binds with much greater affinity to MT-1 MMP and MT-3 MMP, displaying K_i values of 0.07 and 0.17 nM, respectively (*26*). Among these four enzymes, the residence times varied from 7 min (for MMP-9−TIMP-2) to 1.4 h (for MT-1 MMP−TIMP-2). Different TIMPs also appear to regulate different MT MMPs based on variations in residence time among the complexes. Zhao et al. (*26*) compared the binding of TIMP-2, TIMP-3, and TIMP-4 to MT-1 MMP and MT-3 MMP. TIMP-2 was found to be the highest-affinity inhibitor of MT-1 MMP, with a K_i value of 0.07 nM, while TIMP-3 was the best inhibitor of MT-3 MMP ($K_i = 0.008$ nM). Among the six combinations of enzyme and inhibitor pairs, the values of k_{on} were virtually indistinguishable, ranging only from 1.27 to 3.41 $\times$ 10^6 M^{-1} s^{-1} (i.e., a range of <3-fold). What distinguished these complexes from one another were the residence time values, which ranged from 0.46 to 9.7 h. The residence time differences noted in this paper led the authors to suggest that TIMP-3 is the most likely candidate to act as an MT-3 MMP inhibitor under physiological conditions.

Processivity in Biosynthetic Polymerase Reactions. The synthesis of a biopolymer (e.g., proteins and nucleic acids) requires multiple steps of covalent bond formation to produce a polymeric chain from individual components. Reactions of this type require high fidelity and specificity to ensure that the resulting polymer has the correct ordering of components along the polymer. Additionally, these reactions are inherently disfavored, due to the large entropy loss associated with polymer formation. Hence, enzymes that catalyze these reactions must not only confer the appropriate level of specificity of reaction but also energetically compensate for the entropic cost of reaction. One enzymatic mechanism for overcoming this cost is termed processivity. Processive enzymes catalyze multiple rounds of reaction while retaining the substrate in a quasi-stable binary complex. In the case of polymerases, this means that the nascent polymer chain is built up, one unit at a time, while complexed to the polymerase enzyme. This requires the polymerase enzyme to form a stable complex with the nascent polymer, but at the same time be sufficiently dynamic to accommodate the changing specificity of reaction required for chain extension. To facilitate this delicate balance between complex stability and reaction dynamics, polymerases are often found to be composed of multiple protein complexes, in which different proteins confer different characteristics to the overall catalytic mechanism.

The bacteriophage T4 DNA polymerase exemplifies well many of these concepts. The replication of duplex DNA requires a multistep enzymatic pathway that involves assembly of a functional enzyme complex for DNA synthesis, unwinding of a double-stranded DNA template and its coupling to synthesis of complementary RNA primers on the ssDNA lagging strand, and DNA synthesis on leading and lagging strand templates (*27*). These enzymatic reactions have all the requirements described above for catalysis of polymerase reactions with a high degree of fidelity but also require highly processive DNA synthesis on the leading strand with concurrent discontinuous synthesis of small DNA fragments (Okazaki fragments) on the lagging DNA strand. The enzyme complex from T4 bacteriophage has been characterized in detail by Benkovic and colleagues (*27*) and is known as the T4 replisome. It is composed of eight types of proteins: polymerase (gp43), clamp (gp45), clamp loader proteins (gp44 and gp62), helicase (gp41), helicase accessory protein (gp59), primase (gp61), and ssDNA binding protein (gp32) (*28*). The polymerase holoenzyme, composed of clamp protein and polymerase, catalyzes DNA synthesis. In the current model, during DNA replication the T4 replisome contains two polymerase holoenzymes, each simultaneously catalyzing either leading or lagging strand DNA synthesis (*29*).

To achieve the required processivity for DNA replication, the polymerase holoenzyme bound to the DNA leading strand has a residence time of 8−16 min [consistent results reported by Benkovic (*28, 30*) and Alberts (*31*)], similar to the time required to replicate the entire 172 kb T4 genome, which is approximately 15 min (*32*). If the holoenzyme−template residence time were substantially longer than the time for T4 genome replication, DNA replication would be less efficient. Alternatively, if the residence time were shorter, it would diminish processivity, require additional energy, and could compromise fidelity. Catalysis by T4 polymerase alone is distributive; the protein−DNA complex freely dissociates and is recruited from solution during DNA replication with a residence time of <1 s (*33*). Hence, the long residence time required for processivity is provided by the second protein in the holoenzyme, the clamp, which interacts with the polymerase and tethers it to the primer−template junction. Similar to other polymerase reactions, the clamp does not possess an unchanging set of binding interactions with the template but rather moves along the template without dissociating after each round of nucleotide incorporation. The movement of the holoenzyme along the template without clamp dissociation has been termed "dynamic processivity" (*30*). The second polymerase holoenzyme in the T4 replisome catalyzes lagging strand DNA synthesis. This holoenzyme possesses the same inherent processivity observed with the leading strand holoenzyme. However, it must dissociate rapidly from and reassociate with the DNA template repeatedly to allow for efficient Okazaki fragment synthesis. Interestingly, it has been shown that the clamp dissociates rapidly from the lagging strand DNA template and exogenous clamp is recruited for a new round of Okazaki fragment synthesis, whereas the same polymerase is recycled for multiple rounds of lagging strand synthesis (*34*). The resulting shorter residence time of the clamp involved in lagging strand DNA synthesis is required for a rate of lagging strand DNA synthesis similar to that on the leading strand.

RESIDENCE TIME IN DRUG−TARGET INTERACTIONS AND PHARMACOLOGY

The preceding discussion demonstrates the impact of residence time on the biological function of protein−protein

Table 2: Residence Times for Selected Pharmacological Agents (Ligands) on their Target Receptors

receptor	ligand	residence time (τ)[a]	refs	indication
enoyl-acyl carrier protein reductase, *E. coli*	triclosan	1.4 h	*46*	bacterial infection, topical
ribosome complex, *E. coli*	erythromycin	1.6 min	*47*	bacterial infection, systemic
	josamycin	92 min		
HIV-1 protease	nelfinavir	1.1 h	*37*	human immunodeficiency virus type 1 (HIV-1) infection
	lopinavir	1.7 h		
	darunavir	>340 h (>14 days)		
histamine H1 receptor	desloratidine	>8.7 h	*48*	allergic conditions (antihistamine)
angiotensin AT1 receptor	candesartan	2.7 h	*49*	hypertension
	telmisartan	1.9 h		
M3 muscarinic receptor	tiotropium	11 h (1)	*50* (1)	chronic pulmonary disease
		48 h (2)	*51* (2)	
dipeptidyl peptidase IV	vildagliptin	6.6 min	*41*, *42*	type 2 diabetes
	saxagliptin	306 min (5.1 h)		
thrombin	hirudin	14 h	*52*−*54*	thrombosis for patients with heparain-induced thrombocytopenia, deep venous thrombosis, thromboprophylaxis in atrial fibrillation
	lepirudin	16 h		
	argatroban	3 s		
	melagatran	52 s		
serotonin S2 receptor	ritanserin	3.9 h	*55*	bipolar disorder, anxiety, depression
D2 dopamine receptor	haloperidol	58 min	*44*, *56*	schizophrenia
	chlorpromazine	58 min		
	clozapine	0.7 min		
	quetiapine	0.4 min		
epidermal growth factor receptor (EGFR)	lapatinib	7.2 h	*57*	HER2+ breast cancer
heat shock protein 90 (Hsp90)	geldanamycin	6.6 h	*58*	melanoma, breast, prostate, hematologic, and thyroid cancers (close analogues in clinical trials)
neurokinin 1 receptor	aprepitant	3.6 h	*59*	chemotherapy-induced nausea and vomiting
steroid 5α-reductase	finasteride	>43 days (type 1 enzyme), 20 days (type 2 enzyme)	*60*	benign prostatic hyperplasia, prostate cancer, and androgenetic alopecia
gonadotropin-releasing hormone receptor (GnRH)	sufugolix	4.0 h	*61*, *62*	endometriosis, benign prostatic hyperplasia
	NBI 42902	6.2 h		

[a] Residence times were calculated from values of k_{off} or $t_{1/2}^{diss}$, reported in the literature, using the equations defined in Table 1. Note that the units or "approximately" residence time vary among the entries in this column.

complexes, and this suggests that residence time can serve as an important optimization criterion for biopharmaceuticals, such as antibodies, soluble receptors, and the like. Similarly, the residence time of a small molecule drug with a specific target macromolecule represents an important metric for drug optimization, both in terms of duration of efficacy *in vivo* and target selectivity (*10*). It is worth noting in this context that the process of small molecule ligand dissociation from a protein can be considered to be a special case of localized protein unfolding and like other protein folding−unfolding processes will require the system to overcome a sizable energy barrier to attain the unfolding transition state (*35*). For ligand dissociation, this energy barrier is likely to be almost exclusively enthalpic, as the dissociation requires increased mobility for the protein and ligand and will thus be associated with a favorable entropy component. Thus, enhancing the enthalpic contributions of protein−ligand interactions would likely favor slower dissociation and, hence, a longer residence time. Enthalpic contributions to protein−ligand interactions are familiar to medicinal chemists and often dominate the development of structure−activity relationships during the iterative process of drug optimization.

As the application of residence time to drug optimization has recently been reviewed, we will restrict our discussion here to some of the key advantages of long residence time drugs in human medicine. Table 2 provides a representative list of drugs, for various clinical indications, for which long residence time has been associated with biological and/or clinical benefit.

HIV protease inhibitors were first approved in 1995 and have been an essential component of antiretroviral therapy that has resulted in a dramatic decline in morbidity and mortality associated with human immunodeficiency virus type 1 (HIV-1) infection (*36*). HIV protease inhibitor drugs, as exemplified by nelfinavir and lopinavir, have been determined to possess moderately long residence times of ~1 h (*37*). Despite the effectiveness of these agents, the emergence of multidrug resistant HIV that is cross-resistant to multiple HIV protease inhibitor drugs may limit the future utility of these agents (*38*). Darunavir is the most recently FDA-approved HIV protease inhibitor drug, receiving accelerated approval in 2006. This agent has been reported to be distinct from other HIV protease inhibitor drugs because of its excellent antiviral activity against both wild-type and protease inhibitor-resistant HIV and its very high genetic barrier to the development of resistance in cell culture (*39*). Another distinguishing feature of darunavir is its extremely long enzyme−inhibitor residence time of >14 days, essentially irreversible as a pharmacological agent (*37*). The resultant long durability of target inhibition, well beyond its

15 h plasma half-life in patients, is proposed to be an important factor in the high genetic barrier to resistance development.

Another type of pharmacological agent, exemplified in Table 2, is the group of human dipeptidyl exopeptidase IV (DPP IV) inhibitors. DPP IV inhibitors are a promising new class of drugs for the treatment of type 2 diabetes, two of which, vildagliptin and sitagliptin, have been recently approved (*40*). Both drugs are reversible, competitive inhibitors of human dipeptidyl exopeptidase. Vildagliptin has been described as a slow-binding inhibitor, though it possesses a relatively short residence time of 6.6 min (*41*) (the inhibition kinetics of sitagliptin have not yet been reported). More recently, saxagliptin entered phase III clinical trials for type 2 diabetes treatment. This latter drug is a competitive, covalent, slowly reversible inhibitor of DPP IV and has been reported to possess a much longer residence time of 5 h (*42*). Notably, it was reported that saxagliptin demonstrates an extended duration of action in a rodent model measuring pharmacodynamic and functional end points (*43*). The authors speculate that this may be due in part to the kinetics of binding of saxagliptin to DPP IV, though it remains to be demonstrated that this observation will translate into a clinical benefit.

Effect of Residence Time on Pharmacodynamic−Pharmacokinetic Relationships. In the closed systems that are commonly used to study receptor−ligand interactions in the laboratory, the equilibrium dissociation constant serves as a useful measure of the affinity of the receptor−ligand complex. This thermodynamic value is directly influenced by the value of k_{off}, as described in Table 1, so that in situations where k_{on} is relatively invariant, there is a strong correlation between K_d (or K_d*) and k_{off}. In the open system of the human body, however, efficacy and the duration of efficacy (pharmacodynamics) are no longer dependent on affinity, *per se*, but continue to be influenced by the residence time of the binary complex, along with the temporal dependence of drug concentration in systemic circulation (i.e., pharmacokinetics), its tissue distribution and absorption, and other physiological factors.

When an oral drug is ingested, it is absorbed from the intestines into the blood over some time course, typically reaching maximal exposure (C_{max}) within 30 min to several hours. The concentration of the drug in the blood diminishes over time as the drug is distributed to the tissues and is metabolized and eliminated from the body. Figure 2 illustrates a simplified pharmacokinetic time course for systemic exposure to an orally dosed drug. In this illustration, the drug is absorbed and reaches a C_{max} of 500 nM ~1 hr after dosing. From this point, the systemic concentration of drug declines in a monoexponential manner, with an elimination half-life of 5 h. Over this time course, the pharmacological efficacy of the drug will depend on the fractional occupancy of the target receptor at each time point. As pointed out by Vauquelin and Van Liefde (*6*), the time course of receptor fractional occupancy by drug will parallel that of the systemic drug concentration for drugs with residence times that are short relative to their pharmacokinetic half-life. When, however, the residence time of the drug−target complex is long, a significant level of receptor occupancy, and hence pharmacological efficacy, can be sustained even at time points when the systemic level of drug has diminished significantly. To illustrate this, we have simulated the time course of receptor occupancy for three drugs in Figure 2. For this simulation, we assume that all three drugs have identical pharmacokinetic properties (*vide supra*) and that all three inhibit their common target by a two-step, induced-fit mechanism. For such a mechanism, the fractional occupancy of the receptor at any given time ($[fo]_t$) will depend on the concentration of drug available at that time point ($[I]_t$) and on the kinetic rate constants k_1−k_4 as follows (*10*):

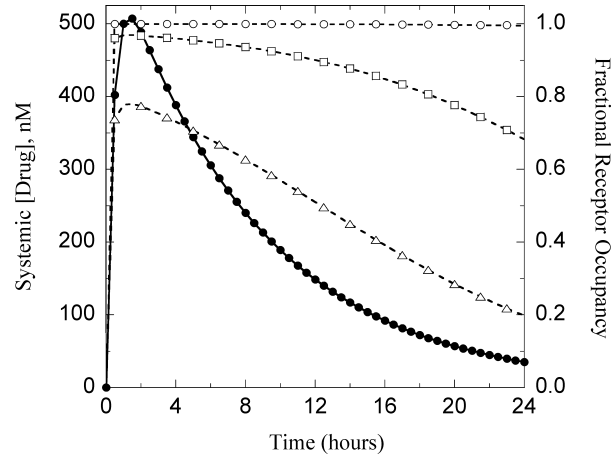

FIGURE 2: Simulated pharmacokinetics and fractional receptor occupancy as a function of time after oral dosing for three drugs that bind, via a two-step, induced-fit mechanism (mechanism B in the text), with different residence times to a common target receptor. The filled circles represent the concentration of each drug in systemic circulation at the indicated times after oral dosing. The empty circles represent the fractional receptor occupancy, at the indicated times after oral dosing, for a drug with a residence time of 116 days. The squares represent the fractional receptor occupancy, at the indicated times after oral dosing, for a drug with a residence time of 2.8 h. The triangles represent the fractional receptor occupancy, at the indicated times after oral dosing, for a drug with a residence time of 17 min. Other values used in these simulations are as described in the text.

$$[fo]_t = \frac{[I]_t}{[I]_t + \left(\frac{k_2}{k_1 + \frac{k_1 k_3}{k_4}}\right)} \quad (2)$$

In our simulation, we have fixed the values of k_1−k_3 for all three drugs at 1×10^6 M^{-1} s^{-1}, 1 s^{-1}, and 6×10^{-3} s^{-1}, respectively. The only parameter that differs among the three drugs in this simulation is the value of the reverse isomerization rate constant, k_4, which was set at 1×10^{-6}, 1×10^{-4}, and 1×10^{-3} s^{-1} for the three drugs, respectively. These values yield residences times of 116 days, 2.8 h, and 17 min, respectively. The impact of these changes on the duration of the effect of the drug is clearly illustrated in Figure 2. Of course, this simulation is a gross oversimplification of the complex, multifactorial nature of drug pharmacokinetics and efficacy *in vivo*. Nevertheless, these simulations demonstrate clearly the potential clinical advantages of long residence time drugs for durable pharmacological efficacy.

Residence Time and Drug Selectivity and Safety. Drug optimization involves more than optimization of molecular interactions between the drug and its macromolecular target. While these interactions are assumed to be the basis of

pharmacological efficacy, an equally important consideration in drug development is safety. Much, but certainly not all, drug toxicity can be attributed to collateral modulation of macromolecules other than the intended target of the drug; such adverse effects are commonly called off-target toxicity. Hence, target selectivity is commonly viewed as a critical component of preclinical drug optimization. Target selectivity is commonly quantified by the ratio of the IC_{50} or K_d values of a drug for a collateral target to the value for the primary target of interest [e.g., $K_{d(collateral)}/K_{d(target)}$]. However, this quantitation of target selectivity is only adequate in the context of a closed system; it does not reflect the temporal changes in primary and collateral target occupancy that occur in an open system. Returning to the simulations presented in the preceding section, we now consider that these simulations no longer reflect three distinct drugs acting on a common target but instead a single drug interacting with three different receptors. We can immediately appreciate from Figure 2 that target selectivity is not a static value *in vivo* but rather evolves with time over the course of treatment, depending on the residence time of the drug complexed to its primary and collateral receptors. The best measure of true target selectivity, then, would need to reflect the ratio of the integrated receptor occupancy over time for the collateral and primary receptors. Stated differently, *in vitro* kinetic data on residence time for a drug complexed to the primary and collateral targets provide a better understanding of the behavior of the pharmacological agent in an open biological system. A drug that displayed a long residence time for its primary target and short residence times against collateral receptors would have a high degree of target selectivity over the course of dosing, and this would likely translate into a better safety profile. On the other hand, a drug that displayed a long residence time against a collateral receptor could result in significantly more safety issues. This latter point may be particularly important in assessing the potential for drug−drug interactions in patients due to the collateral inhibition of drug-metabolizing enzymes, such as the cytochrome P450 family (*10*).

CONCLUDING REMARKS

In this brief review, we have introduced the concept of residence time for receptor−ligand complexes and provided quantitative definitions that can form the basis of laboratory determinations of residence time. We have described the physiological and pharmacological importance of residence time for receptor−ligand complexes in the context of the open systems that constitute *in vivo* biology. Much of our discussion has focused on the advantages of a long residence time for a receptor−ligand complex that can translate into a durable biological effect on receptor function. However, a long residence time is not a universally desired effect in biology or pharmacology. We have described, for example, how a long residence time for a collateral receptor can lead to safety concerns for a candidate drug. Thus, long residence times against collateral receptors are to be avoided in an effort to ameliorate off-target-based toxicity. Not all toxicity, however, is due to collateral modulation of nontarget receptors. In some cases, engagement of a single receptor can lead both to pharmacological activity and toxic effects, and sometimes, the difference between efficacy and toxicity can be linked to sustained ligand occupancy and, hence, residence time. This is the case, for example, for antagonists of the D2 dopamine receptor which are used clinically as antipsychotics (see Table 2). Early examples of this drug class, such as haloperidol and chlorpromazine, are high-affinity antagonists of the receptor with residence times in excess of 30 min. In addition to their pharmacological efficacy, these drugs elicited a number of side effects, including extrapyramidal (Parkinson-like) symptoms and prolactinemia. More recently, D2 receptor antagonists have been introduced with much shorter residence times (16−30 s), and this appears to have been an important mechanism for attenuating the negative side effects of these drugs [for more details, see Kapur (*44*) and Seeman (*45*)].

Whether long residence time is a desirable or undesirable characteristic of a receptor−ligand pair will depend ultimately on the effect of ligand engagement on biological activity and the physiological context of the receptor−ligand system. In either case, a quantitative assessment of this parameter is an important part of the overall evaluation of receptor−ligand interactions throughout biochemistry and, perhaps particularly, in drug discovery and pharmacology.

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