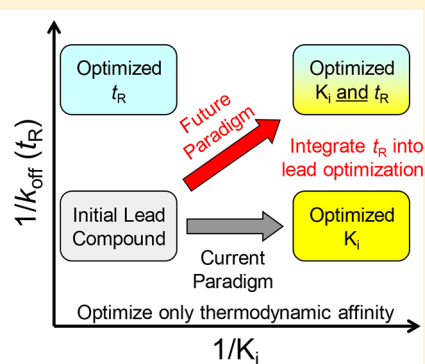
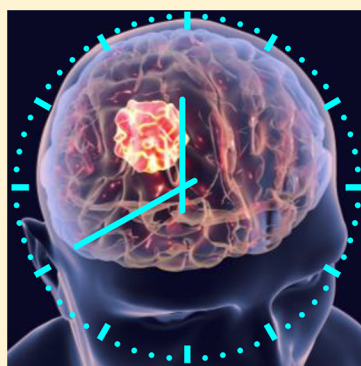


Drug–Target Kinetics in Drug Discovery

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ABSTRACT: The development of therapies for the treatment of neurological cancer faces a number of major challenges including the synthesis of small molecule agents that can penetrate the blood-brain barrier (BBB). Given the likelihood that in many cases drug exposure will be lower in the CNS than in systemic circulation, it follows that strategies should be employed that can sustain target engagement at low drug concentration. Time dependent target occupancy is a function of both the drug and target concentration as well as the thermodynamic and kinetic parameters that describe the binding reaction coordinate, and sustained target occupancy can be achieved through structural modifications that increase target (re)binding and/or that decrease the rate of drug dissociation. The discovery and deployment of compounds with optimized kinetic effects requires information on the structure–kinetic relationships that modulate the kinetics of binding, and the molecular factors that control the translation of drug–target kinetics to time-dependent drug activity in the disease state. This Review first introduces the potential benefits of drug–target kinetics, such as the ability to delineate both thermodynamic and kinetic selectivity, and then describes factors, such as target vulnerability, that impact the utility of kinetic selectivity. The Review concludes with a description of a mechanistic PK/PD model that integrates drug–target kinetics into predictions of drug activity.

KEYWORDS: Kinetic selectivity, target vulnerability, target occupancy, drug–target residence time, PK/PD modeling, therapeutic window, blood brain barrier, CNS cancer

INTRODUCTION

The treatment of primary infiltrative and secondary metastatic CNS tumors requires the development of drugs that can penetrate the blood-brain barrier (BBB), a selectively permeable barrier composed of epithelial cells held together by tight junctions that is rich in efflux transporter proteins such as P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp).¹ The BBB severely limits the ability of many drugs to penetrate into the brain and is a major impediment to the development of new CNS therapies. For instance, given the success at developing drugs that target kinases in peripheral tumors, it is salutatory that no kinase inhibitor has yet received approval for the treatment of primary CNS cancers.² In addition, brain metastasis is a common resistance mechanism during treatment of peripheral tumors due to the inability of drugs to penetrate the BBB. Thus, since drug exposure is likely to be lower in the brain than in systemic circulation, strategies should be adopted that involve the design and synthesis of compounds that remain bound to their targets even when drug concentration is low. However, the general reliance in drug discovery

programs on in vitro assays performed at constant drug concentration limits the identification and progression of compounds that display kinetic effects. This knowledge gap will also impact the development of covalent inhibitors since the benefit gained from prolonged target engagement depends on factors such as target vulnerability that can only be assessed using time-dependent assays.

Drug discovery is predicated on the identification and optimization of drug leads through a series of in vitro experiments that are usually performed at constant concentration. The quantitative metrics that result, such as the IC₅₀ values for engagement of the purified target or for activity in a cell-based assay, are used to select and prioritize lead compounds, make assessments about the possibility for off-target effects that impact the therapeutic index, and ultimately to predict drug activity.

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However, equilibrium parameters are not able to fully account for time-dependent changes in target engagement in the dynamic environment of the human body where drug (and target) concentrations fluctuate. Instead, both the thermodynamics and kinetics of drug-target interactions must be utilized to fully account for time-dependent changes in target engagement. The role of drug-target kinetics in drug discovery has been discussed in a number of reviews and opinions,^{3–11} and key concepts include potential mechanisms that modulate the rates of drug-target complex formation (k_{on}) and breakdown (k_{off}),^{12–16} including the development of covalent inhibitors,¹⁷ and the role that drug-target residence time ($1/k_{\text{off}}$), k_{on} , and pharmacokinetics play in dictating target engagement.^{18–26} In addition, the role of binding kinetics has been explored in forward-thinking programs, such as the K4DD Innovative Medicines Initiative.²⁷ The present Review reiterates some of the basic concepts that govern drug-target interactions and shows that access to the on and off rates for formation and breakdown of the drug-target complex provides an additional dimension of information that can be used to prioritize drug leads based on kinetic selectivity. Subsequently it is shown that the translation of kinetic effects to time-dependent changes in drug activity depends on target vulnerability which directly impacts kinetic selectivity. The review concludes with a discussion of pharmacokinetic/pharmacodynamic (PK/PD) models that integrate drug-target kinetics into predictions of drug activity in order to facilitate the prospective use of in vitro kinetic data.

■ THE THERMODYNAMICS AND KINETICS OF DRUG-TARGET INTERACTIONS

Drug-target complex formation occurs because the complex is more thermodynamically stable than free unbound drug and target: thus, thermodynamics provides the driving force for drug binding. However, the value for the equilibrium constant that describes binding provides no information on the rate at which the complex forms and breaks down. Instead, the on (k_{on}) and off (k_{off}) rates for drug binding are controlled by the difference in free energy between the relevant ground and transition states on the binding reaction coordinate (Figure 1).

Since k_{on} and k_{off} depend on the difference in free energy between the ground and transition states on the binding reaction coordinate, efforts to improve drug potency may have unpredictable effects on the kinetics of drug-target complex formation and breakdown. Potency is normally used as a descriptor of affinity, and thus an increase in potency is associated with an increase in the affinity of the drug-target interaction quantified by a decrease in the K_{d} or IC_{50} value. However, the impact of stabilizing the E-I ground state on the kinetics for binding depends on whether the transition state is affected.⁸ Several scenarios can be envisaged, the simplest of which is that stabilization of E-I has no effect on the transition state. In this case a decrease in K_{d} will lead to a decrease in k_{off} with no change in k_{on} . In other words, a more potent compound will have a slower off-rate. Alternatively, if the changes in compound structure that lead to an increase in affinity also result in equal stabilization of the transition state, then the increase in potency will have no effect on the rate at which the drug dissociates from the target. In addition, a third scenario can be envisaged where two molecules have identical affinities for the target (the same K_{d} or IC_{50} values) but different k_{on} and k_{off} values. Importantly, any differences in k_{on} and k_{off} values, either between two molecules binding to the same target or a

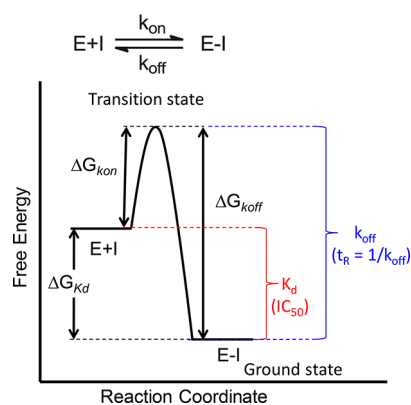


Figure 1. Reaction coordinate for a one-step binding event. Target (E) and drug (I) binding leads to the drug-target complex (E-I). The driving force for binding is given by the difference in free energy between E+I and E-I ($\Delta G_{K_{\text{d}}}$). Experimental measurements of the equilibrium dissociation constant K_{d} , or parameters such as IC_{50} values, provide a quantitative estimate of the thermodynamics for binding. The rate at which the drug-target complex forms (k_{on}) and dissociates (k_{off}) is given by the difference in free energy between the respective ground states (E+I or E-I) and the rate-limiting transition state ($\Delta G_{k_{\text{on}}}$ and $\Delta G_{k_{\text{off}}}$). $\Delta G_{K_{\text{d}}}$ is related to K_{d} by the relationship $\Delta G^{\circ} = -RT \ln K$. Assuming that parameters such as the transmission coefficient are the same for two drug molecules, then the difference in free energy for the rate of complex dissociation of the two molecules can be given by $\Delta \Delta G_{k_{\text{off}}} = -RT \ln(k_{\text{off}}^1/k_{\text{off}}^2)$. The lifetime of the drug-target complex is often quantified by the residence time, t_{R} , where $t_{\text{R}} = 1/k_{\text{off}}$.⁵ The figure shows a simple one-step mechanism, although in many cases slow-binding inhibitors operate through a two-step induced-fit mechanism.^{13,15,28–31}

molecule interacting with two different biological molecules (e.g., on and off-target proteins), will not be revealed by approaches that only evaluate compound affinity (potency).

Two important misconceptions abound. First, it is often assumed that an increase in potency will result in a decrease in k_{off} . It may do, but it does not have to. Although there are many examples of long residence time compounds, kinetic data for structurally related analogs are often not available, preventing an analysis of whether or not residence time is driven by affinity. Examples where changes in structure within a compound series lead to a decrease in both IC_{50} and k_{off} include inhibitors of *Pseudomonas* LpxC,²¹ human protein methyltransferase DOT1L,³² and CDK8/CycC.³³ In addition, if stabilization of E-I also results in stabilization of the transition state, then once the theoretical limit for k_{on} is reached, which is the second order rate constant for encounter of drug and target ($10^9 \text{ M}^{-1} \text{ s}^{-1}$), any further increase in affinity must lead to a reduction in the off-rate. A simple calculation reveals that the residence time of a 1 pM drug on the target must be at least 12 min whereas a 1 fM drug will have a residence time of at least 11.5 days. However, there are also examples where changes in affinity and off-rate are disconnected, which are particularly relevant where affinities are in the micromolar to nanomolar range: a 1 nM drug may only have a residence time of 1 s on the target. For example, the quinazoline-based inhibitors gefitinib and lapatinib have $K_{\text{i}}^{\text{app}}$ values for EGFR of 0.4 and 3 nM, respectively, but while gefitinib has a residence time of <14 min, lapatinib has a residence time of 430 min.³⁴ Other examples include antagonists of the muscarinic M3 receptor,³⁵ antagonists of the chemoattractant receptor-homologous

molecule CRT α 2/DP2,³⁶ and inhibitors of p38 α MAP kinase.³⁷ The second major misconception is that if a compound has similar IC₅₀ values for two different proteins, the drug-target and an off-target protein associated with unwanted side-effects, then the compound has no selectivity. Indeed, the compound has no *thermodynamic* selectivity, but if the k_{on} and k_{off} values differ between the two targets, it can still have *kinetic* selectivity. (Text Box 1). This is very important given the implicit relationship between selectivity and therapeutic index.

KINETIC SELECTIVITY

The relative affinity of a compound for the target and for any known off-target proteins is commonly used as a metric for compound selectivity and the potential of the molecule for causing unwanted side-effects. As noted above, this definition of selectivity is actually *thermodynamic* selectivity since it is based on equilibrium binding experiments (e.g., IC₅₀ values). Of course the ability to determine selectivity depends on knowledge and availability of known off-target proteins and, for example, kinase inhibitor discovery programs often determine compound activity toward a panel of kinases to assess the potential for off-target effects. However, *thermodynamic* selectivity may not map with *kinetic* selectivity, and indeed as noted above, *kinetic* selectivity can still exist even in the absence of *thermodynamic* selectivity.^{21,23,38–41}

Figure 2 shows a simulation where a compound binds to four targets with the same K_d but different on and off rates,

including a target in which a covalent complex is formed ($k_{\text{off}} = 0$). The panels show how the occupancy of the targets change with time as a function of drug concentration (pharmacokinetics) assuming that no target turnover occurs. When compound eliminates with a half-life of 5 h (Figure 2A and C), all four targets reach >95% occupancy even at the lower initial drug dose (Figure 2C). In addition, selectivity between the three targets to which the compound binds reversibly (Targets 1–3) only occurs after more than 12 h. In contrast, if the compound half-life is only 1 h (Figure 2B and D), then there is a high degree of selectivity: for example, at 12 h for the higher drug dose (Figure 2C), Target 1 is only 5% occupied while Targets 2 and 3 are 54% and 83% occupied, respectively. Thus, despite the lack of *thermodynamic* selectivity, the compound demonstrates *kinetic* selectivity between the targets. In addition to providing an explicit example of kinetic selectivity, three additional points may be drawn from this analysis. First, the relationship between binding kinetics and drug pharmacokinetics plays a fundamental role in controlling time dependent occupancy. Second, a covalent inhibitor can in principal maximize the impact of kinetic selectivity. Third, when the compound eliminates more rapidly, Target 3 only reaches 70% occupancy at the lower dose (Figure 2D). Since K_d is kept constant, a decrease in off-rate must also lead to a reduction in the on-rate. The faster elimination results in lower peak compound concentration and, since k_{on} is a second order rate constant, lower occupancy of the target. Thus, the level of

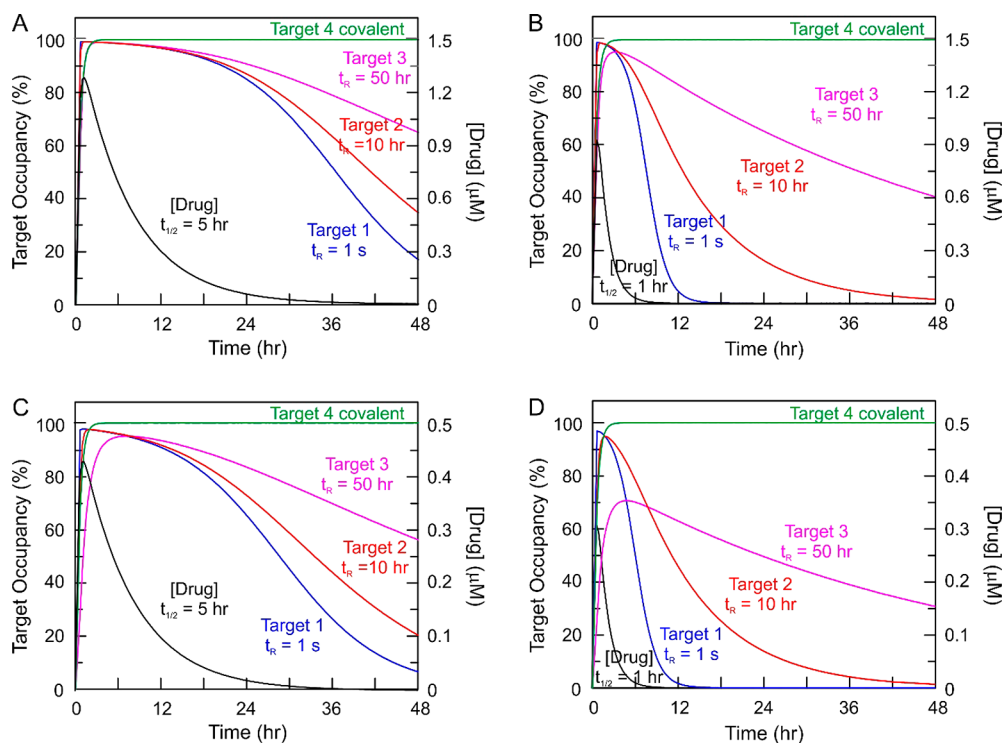


Figure 2. Time-dependent target occupancy: kinetic selectivity. A compound is assumed to bind reversibly to three targets with the same thermodynamic affinity (10 nM) but have different residence times on the three targets: Target 1, 1 s; Target 2, 10 h; and Target 3, 50 h). In addition, the compound is assumed to bind covalently to a fourth target (Target 4). Target occupancy has been simulated using Kintek,^{44,45} assuming either a 1.5 μM (A and B) or 0.5 μM (C and D) dose of compound that is absorbed with k_a 3 h^{-1} but eliminated with two different rates, k_e 0.139 h^{-1} ($t_{1/2}$ 5 h) (A and C) or k_e 0.69 h^{-1} ($t_{1/2}$ 1 h) (B and D). Reversible binding is assumed to occur via a one-step mechanism with the following on and off-rates. Target 1: k_{on} 100 $\mu\text{M}^{-1} \text{s}^{-1}$, k_{off} 1 s^{-1} . Target 2: k_{on} $2.78 \times 10^{-3} \mu\text{M}^{-1} \text{s}^{-1}$, k_{off} $2.78 \times 10^{-5} \text{s}^{-1}$. Target 3: k_{on} $5.56 \times 10^{-4} \mu\text{M}^{-1} \text{s}^{-1}$, k_{off} $5.56 \times 10^{-6} \text{s}^{-1}$. For Target 4, it is assumed that the compound binds in a two-step mechanism in which the initial rapid binding of the compound to the target, defined by k_{on} 100 $\mu\text{M}^{-1} \text{s}^{-1}$ and k_{off} 1 s^{-1} is followed by a second step with k_{inact} $5.56 \times 10^{-4} \text{s}^{-1}$ leading to the covalent drug-target complex. In each case the target concentration is fixed at 1 nM (i.e., no target turnover).

target occupancy is a function of both k_{on} and k_{off} , as well as drug concentration. In addition, the analysis in Figure 2 assumes a target concentration of 1 nM which is 10-fold lower than the K_d (10 nM). However, if the K_d is similar to the target concentration, then binding to the target can modify the local drug concentration and hence the pharmacokinetics of the drug (target-mediated drug disposition),^{23,42,43} in a situation that is analogous to tight-binding enzyme inhibition. This effect will become more pronounced as the total drug concentration approaches the concentration of the target. This effect will become more pronounced as the total drug concentration approaches the concentration of the target. Other factors, such as the local accumulation of compounds in the plasma membrane and binding to plasma protein may also contribute to the “micropharmacokinetics” of the drug.²⁰

Box 1. Thermodynamic and Kinetic Selectivity

Selectivity refers to the relative ability of a drug to engage the chosen target compared to off-target macromolecules, and provides valuable insight into the potential for unwanted side effects (i.e., the therapeutic window or therapeutic index). In many cases, selectivity is determined from affinity-based measurements, for example, by comparing IC_{50} values for kinase inhibition in a kinase panel. However, this is actually *thermodynamic selectivity* since IC_{50} values, as well as K_d and K_i values, are determined at constant drug concentration. Since a compound can have the same affinity for two proteins, but different on and off-rates, affinity-based assessments of selectivity provide no insight in to the possibility that a compound may show *kinetic selectivity* between two proteins. In other words, a drug may have the same affinity for two proteins but dramatically different binding kinetics such that the lifetimes of the two drug-target complexes differ by orders of magnitude. The contribution of *kinetic selectivity* to the therapeutic window is intimately related to the time-dependence of drug concentration at the target site (pharmacokinetics, PK), and drugs that eliminate rapidly relative to the lifetime of the drug-target complex will maximize the potential benefit of kinetic selectivity in situations where prolonged occupancy of the target is mitigated. The required PK for long residence time drugs will thus likely be similar to that for covalent drugs where high C_{max} ensures rapid occupancy of the target and fast elimination then maximizes the therapeutic window.¹⁷ In reality the C_{max} only has to be high enough to ensure that physiologically relevant levels of target engagement are achieved, and indeed the relationship between target occupancy and drug efficacy is dictated by the vulnerability of the target (see below). In addition, target turnover will also impact kinetic selectivity, since the rapid synthesis of new target will negate the effects of prolonged target occupancy at low drug concentration. Finally, the rational optimization of *kinetic selectivity* requires knowledge of both the ground and transition states on the binding reaction coordinate, which is important since rational drug design normally only focuses on enhancing affinity through stabilization of the drug–target ground state.

TARGET VULNERABILITY

In Figure 2 it can be seen that target occupancy varies with time based on the kinetics of drug binding as well as drug concentration, and that in some scenarios complete occupancy of the target may not occur. The translation of target occupancy to drug pharmacodynamics depends on the relationship

between occupancy and effect, which in turn depends on target vulnerability, that is, what fraction of target has to be engaged to elicit the desired response (Text Box 2). Low vulnerability targets are those where high levels of occupancy are needed to generate the desired physiological outcome. Conversely, high vulnerability targets require only low levels of occupancy to achieve the desired effect. The specific relationship between occupancy and effect can be captured using a vulnerability function, and in Figure 3 are shown examples of the functions for hypothetical low and high vulnerability targets.

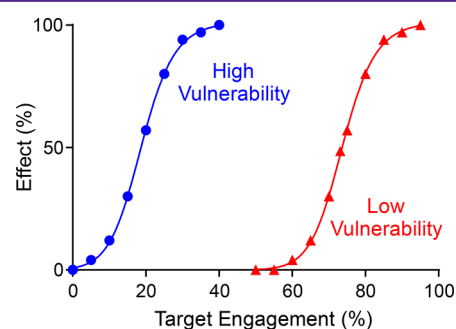


Figure 3. Target vulnerability plots. Vulnerability functions are shown for low (red) and high (blue) vulnerability targets. The vulnerability function is defined by the minimum level of engagement required for any effect to be observed (TO_{min}) and the level of engagement that leads to the maximal efficacy (TO_{max}). The third parameter required to define the function is the Hill coefficient or slope factor that determines the steepness of the effect response between TO_{min} and TO_{max} . For the low vulnerability target, the full physiological effect of the drug requires close to 100% target engagement, whereas only ~35% engagement is needed for the high vulnerability target. The Hill coefficients for the two functions are 4.6 (high) and 16.4 (low).

The term vulnerability is often used in an “all-or-none” context in which targets are defined as vulnerable or not depending on whether or not target engagement results in the desired physiological effect. In Figure 3, this definition is taken one step further where we consider the degree of engagement that is required to generate the desired response. Clearly a low vulnerability target will require relatively higher levels of drug exposure to achieve pharmacologically relevant levels of engagement compared to a high vulnerability target. In addition, low vulnerability targets will be less susceptible to kinetic selectivity since it will take less time for a small fraction of active target to be generated either by drug dissociation or by the synthesis of the small percent of new target required to alleviate the impact of target engagement. Approaches that have been used to infer target vulnerability include methods that reduce the amount of target in the cell, either by genetic knockdown or by directly depleting proteins by inducible degradation.^{46–48} As we show below, cell-based washout experiments can also provide insight into target vulnerability.

Based on the discussion above, we would argue that the degree of target vulnerability must factor into considerations of target “druggability” since presumably it will be easier to “drug” a high vulnerability target compared one that is less vulnerable. It then follows that it is important to identify the molecular factors that influence the target vulnerability function. Under conditions that favor kinetic selectivity, for example, when the rate of drug elimination is rapid relative to the rate of drug–target complex breakdown, then the rate of target turnover will

play a major role in controlling target vulnerability since rapid target resynthesis will alleviate the impact of even a covalent inhibitor once free drug has been removed. In addition, the physiological context of the target and the downstream consequences of target engagement must also play a role in vulnerability. For instance, an enzyme that catalyzes the rate limiting step in a metabolic pathway might be more vulnerable to target engagement compared to other enzymes in the pathway. Furthermore, the length of time that a target must be engaged will also be important: a more vulnerable target might be one where only transitory engagement is needed to trigger a cascade of events that result in the desired pharmacodynamic effect.

■ PROBING TARGET VULNERABILITY: CELL WASHOUT EXPERIMENTS

Although kinetic selectivity can be defined at the level of a purified target, the role that kinetic parameters such as drug-target residence time play in drug activity will be controlled by the relationship between target engagement and time-dependent drug activity. It is thus crucial to evaluate time-dependent effects of drug treatment in more complex biological systems. As we mentioned above, compound activity is often quantified using only IC_{50} values obtained at constant compound concentration. This is also true for measurements of cell-based activity, which are also often only evaluated using experiments performed at constant concentration. Thus, any assessment of kinetic selectivity must include cell-based washout experiments, in which the phenotypic consequences of target engagement are evaluated once drug is “removed” from the system. Such experiments will provide direct insight into target vulnerability and the role that molecular processes such as target turnover play in controlling the coupling between drug-target residence time and time-dependent drug activity. Conceptually the approach is straightforward once a method is in place to remove compound from the media that surrounds the cells. In antibacterial space, the postantibiotic effect (PAE) of a compound is normally assessed by diluting cells exposed to drug into fresh media and monitoring the rate of regrowth.⁴⁹ This approach can be employed for other types of cells that are grown in suspension. Alternative approaches are available for cells that grow on surfaces.

The cell washout experiment is very informative since it provides insight into how long it takes for the level of target engagement to fall to a point where the cell recovers from drug treatment. Intuitively we can see that target vulnerability will be a factor in controlling the length of time it takes for a cell to recover from drug removal since it will take relatively less time for processes such as target dissociation or target resynthesis to generate sufficient active target to alleviate the impact of drug binding to a low vulnerability target compared to a high vulnerability target. Thus, for a compound that has prolonged target engagement due to long residence time or covalent inhibition, the lack of long lasting effects of drug treatment following washout suggests that the target has low vulnerability, at least under the growth conditions that are employed. Conversely, a prolonged phenotypic response following washout would suggest a higher level of target vulnerability. Of course there are caveats: for example, in addition to long residence time, drug rebinding and/or accumulation of drug in the cell or membrane will result in extended target engagement. In addition, prolonged drug effects might also be due to the slow repair of essential processes that were reversibly damaged by drug treatment. Finally, it is important to reiterate a point made

above: the time-dependent effects of drug treatment will depend on the growth conditions. The media used to culture cells in the lab may present a very different environment to that experienced *in vivo*, due to immune pressure or nutrient limitation, which could have a profound effect on target vulnerability in the same way that growth conditions can influence target essentiality. Indeed, the observation of prolonged *in vivo* effects after drug has been eliminated when no time-dependent effects are observed *in vitro* may indicate that the target is more vulnerable *in vivo* than *in vitro*.

Correlations between residence time and cellular washout have been examined in a number of systems. For antagonists of inflammatory protein complement C5a, an increase in residence time was shown to correlate with extended cellular activity following washout.⁵⁰ Conversely, whereas the cellular kinetics of histone acetylation correlated with the kinetics of HDAC inhibition, washout of long residence time benzamide-based HDAC inhibitors did not result in a prolonged phenotypic response.⁵¹ In addition, we have assessed the translation of drug–target residence time to time-dependent antibacterial activity for two structurally related compound series that inhibit the FabI enoyl-ACP reductase, a target in Gram-positive and Gram-negative bacteria, and UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase (LpxC), a target in Gram-negative bacteria. Evaluation of the relationship between residence time and postantibiotic effect (PAE) revealed a steeper correlation for LpxC than FabI suggesting

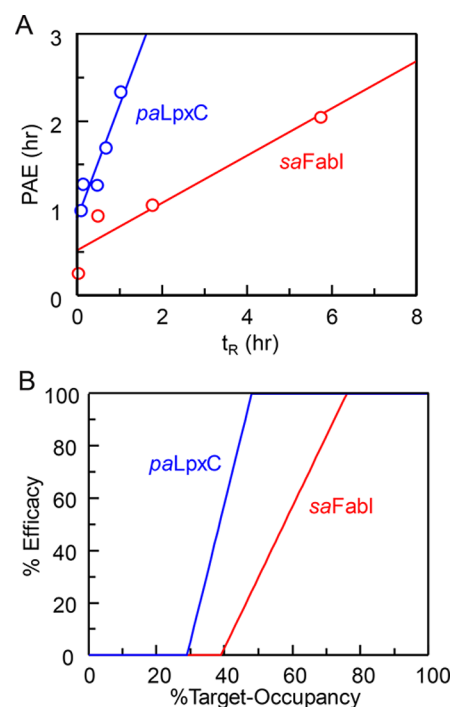


Figure 4. Vulnerability of two antibacterial targets: LpxC and FabI. (A) Correlation between residence time (t_R) and postantibiotic effect (PAE) for two series of antibacterial compounds that target UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase from *Pseudomonas aeruginosa* (*paLpxC*) and the enoyl-ACP reductase from *Staphylococcus aureus* (*saFabI*). (B) Vulnerability functions after global fitting of the PAE data to a PK/PD model that integrates drug-target kinetics into predictions of drug activity. Both plots support the conclusion that *paLpxC* is more vulnerable than *saFabI*.^{21,24} Adapted from ref. 24 with permission from the Royal Society of Chemistry.

Box 2. Target Vulnerability and Cell Washout Experiments

Target vulnerability is the fractional target occupancy required to produce the desired pharmacodynamic (PD) response and is defined by a vulnerability function given by values for TO_{\min} , TO_{\max} , and a Hill coefficient. Low vulnerability targets require high levels of occupancy to achieve the desired PD, whereas high vulnerability targets require lower levels of occupancy. Target vulnerability can be assessed through cell washout experiments where free drug is “removed” from the system by washing or dilution. Prolongation of the phenotypic response to drug treatment following washout can be due to several factors including continued occupancy of the target by the drug. In turn, continued target occupancy may be the result of a slow off rate, or drug rebinding. Correlations between the off rate (drug–target residence time) and a prolonged phenotypic response inform on target vulnerability and the potential for kinetic selectivity to play a role in drug pharmacology. Where possible, the drug–target kinetic data should be obtained at 37 °C since temperature will affect the rate constants for drug binding. In addition, it should be noted that target vulnerability will be affected by environmental factors such as growth conditions, and so target vulnerability in (e.g.) cell culture may be different from that found in vivo.

that LpxC is more vulnerable under the growth conditions employed (Figure 4).^{21,24}

TRANSLATING DRUG-TARGET KINETICS TO PREDICTIONS OF DRUG-ACTIVITY: MECHANISTIC PK/PD MODELS

Pharmacokinetic/pharmacodynamic (PK/PD) models predict the effect time-courses resulting from administration of a drug dose. In order to fully utilize the information gained by a detailed analysis of binding kinetics, we have developed PK/PD models that integrate drug–target kinetics into predictions of

drug activity.^{21,24,26} Essentially this involves the replacement of the Hill receptor binding equation in standard PK/PD models with the full kinetic scheme that describes the drug binding reaction coordinate (Text Box 3). In this way *both the thermodynamics and kinetics* of drug binding can be used to predict target engagement as a function of time and drug concentration. Importantly, the Hill receptor equation assumes that a rapid equilibrium exists between drug and target. However, this assumption can seriously underpredict target engagement in situations where a drug has a long residence time on the target relative to the rate of drug elimination. The PK/PD model was used to successfully predict the *in vivo* activity of a *paLpxC* inhibitor in an animal model of infection (Figure 5),²¹ and also of inhibitors of *saFabI*.²⁴ Significantly, the predicted drug efficacy is much less if a rapid equilibrium is assumed between drug and target. In other words, use of a traditional PK/PD model would mandate much higher drug doses than actually needed. This analysis supports the underlying importance of the drug–target kinetic approach, and particularly the potential benefit of drugs with long residence times: compounds that dissociate slowly from their targets may have prolonged activity at low drug concentration enabling dosing frequency and/or dosing levels to be reduced thereby leading to an increase in the therapeutic window.

IN VIVO TARGET VULNERABILITY: CHEMICAL TOOLS TO QUANTIFY TARGET ENGAGEMENT

The PK/PD model described above uses data from *in vitro* cell washout experiments to optimize the parameters that are required to predict *in vivo* drug activity.^{21,24} This approach, which involves the calculation of target engagement as a function of time and drug concentration, can be improved if *in vivo* target engagement can be directly quantified since this provides direct insight into the relationship between drug binding and efficacy (and indeed also evidence that the drug is

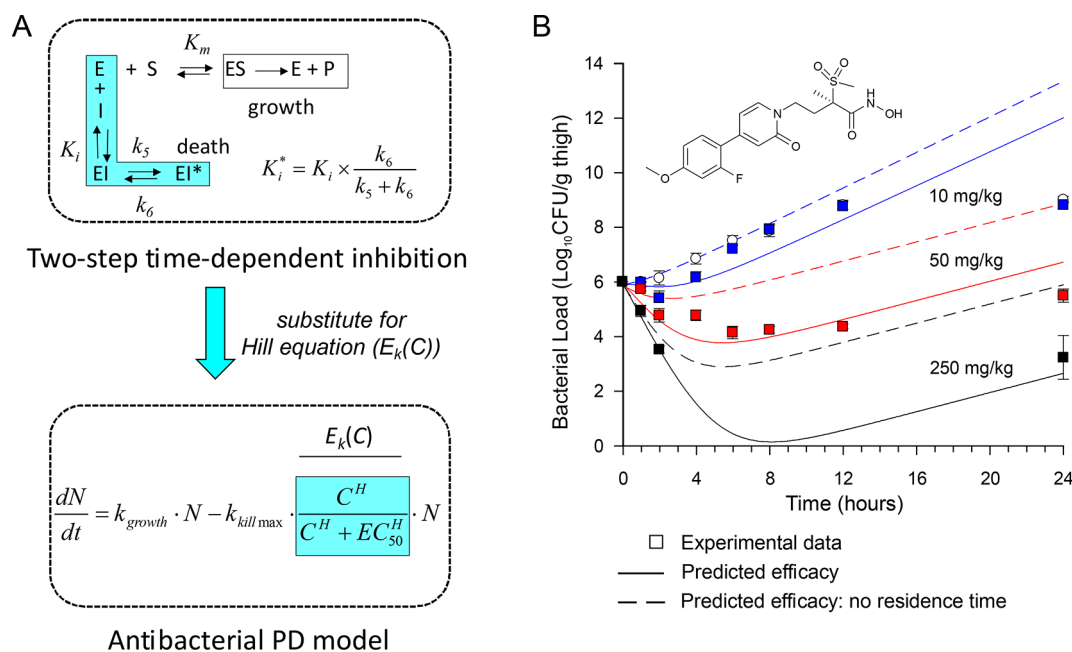


Figure 5. Mechanistic PK/PD model. (A) The kinetic mechanism for two-step time-dependent inhibition replaced the Hill equation in a standard antibacterial pharmacodynamic model. (B) The model was used to successfully predict the efficacy of a *paLpxC* inhibitor in an animal model of infection (solid line). PK/PD modeling assuming rapid equilibrium between drug and target significantly underestimates the observed efficacy (dashed line). Figure adapted from Walkup et al.²¹

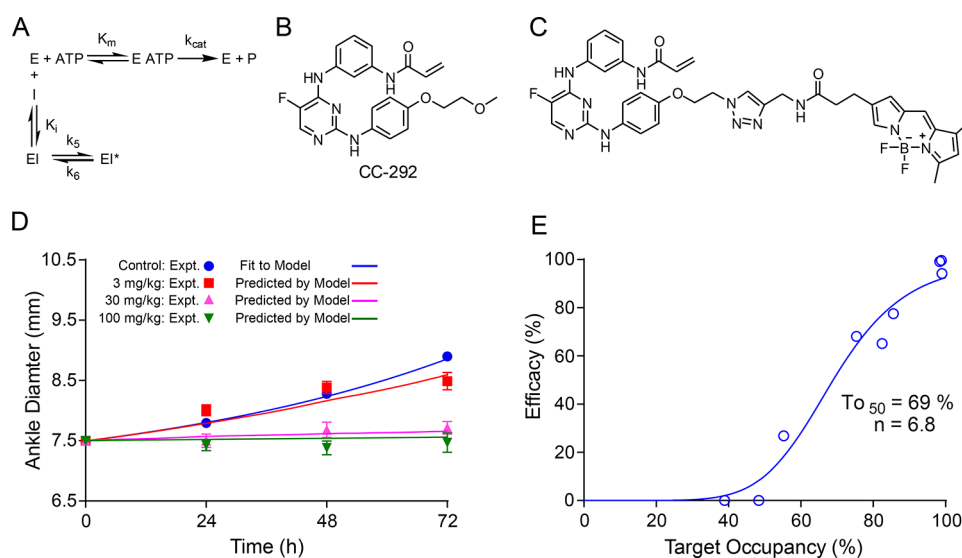


Figure 6. PK/PD model predicts the efficacy of CC-292, a covalent inhibitor of Btk (A) The kinetic scheme for inhibition of Btk by CC-292. Since CC-292 is an irreversible inhibitor, $k_5 = 0$. (B) Structure of CC-292. (C) Fluorescent analogue of CC-292 used to quantify Btk engagement. (D) Predicted and observed efficacy of CC-292 in a rat model of collagen induced arthritis. (E) Vulnerability function for engagement of Btk. Adapted from ref. 26 with permission from the Royal Society of Chemistry.

actually binding to the designated target). In systems that involve covalent inhibition, such as in tyrosine kinases that have a conserved Cys at their active sites, it is possible to develop active site-directed covalent probes to quantify target engagement,⁵² and we have used this approach to analyze the inhibition of the Bruton's tyrosine kinase (Btk) by the covalent inhibitor CC-292.⁵³ Btk is a nonreceptor tyrosine kinase that is a promising target for treating diseases caused by B cell dysregulation, such as B-cell malignancies and autoimmune diseases including rheumatoid arthritis and lupus.^{54–58} CC-292⁵³ as well as drugs such as ibrutinib⁵⁹ contain an acrylamide electrophile that reacts with a conserved Cys (481) in the Btk active site. We synthesized a fluorescent probe based on CC-292 that was used to quantify levels of Btk engagement by CC-292 both in cell culture (Ramos cells) as well as in B lymphocytes obtained from rats dosed with CC-292. Initial values for PK/PD modeling were obtained by quantifying CC-292 binding to Btk in Ramos cells first under equilibrium conditions and then following washout of CC-292 to enable the rate of Btk turnover to be estimated. In addition, the kinetic parameters for Btk inhibition by CC-292 were calculated using the extracellular concentration of CC-292 in plasma (free fraction), thus removing the need to estimate the drug concentration across the cell membrane. Indeed, the ratio of the K_i values determined for cellular Btk compared to purified Btk was 80, suggesting that the CC-292 concentration in the cell was 80-fold lower than the concentration in the media or plasma. Fitting of time-dependent *in vivo* Btk engagement to a PK/PD model that explicitly included target turnover (ρ) and the ratio of [ATP] to the K_m value for ATP ($M = K_m/[ATP]$) provided a set of optimized parameters that were then used to accurately predict the efficacy of Btk in a rat model of collagen-induced arthritis (CIA). This then enabled an explicit evaluation of Btk vulnerability *in vivo* (Figure 6).²⁶ The Btk vulnerability function indicates that >90% occupancy is needed to deliver the maximum efficacy in the rat CIA model, and that levels of engagement below ~50% have no beneficial effect. This suggests that Btk is a relatively low vulnerability target.

Box 3. Mechanistic PK/PD Models That Include Drug-Target Kinetics

A PK/PD model has been developed that integrates both the kinetics and thermodynamics of drug binding into predictions of drug activity. The model calculates target engagement as a function of both drug concentration and time, and then relates the time-dependence of engagement to drug pharmacodynamics. *In vitro* washout experiments are used to generate optimized parameters for enzyme inhibition and target turnover that are then used to predict *in vivo* efficacy. Quantification of target engagement using a covalent probe enables target turnover to be explicitly included in the model and provides direct insight into the vulnerability of the target *in vivo*.

The Btk study demonstrates that a covalent probe can be used to directly determine both TO_{min} and TO_{max} as well as the shape of the vulnerability function. For the reversible inhibitors of *paLpxC* and *saFabI*, estimates for TO_{min} and TO_{max} were obtained by calculating target engagement using the kinetic parameters for enzyme inhibition and then fitting the cellular washout (PAE) data obtained for each compound in the series to the PK/PD model. The vulnerability functions in Figure 4 were then generated by assuming a linear increase in antibacterial activity between TO_{min} and TO_{max} . While this approach can be used to determine the *in vivo* vulnerability functions for *paLpxC* and *saFabI*, as well as other reversible and irreversible inhibitors, approaches that correlate engagement and efficacy have traditionally relied on biomarkers, including the use of positron emission tomography (PET) radiotracers to noninvasively quantify *in vivo* target engagement.^{60–62} Examples of these approaches include the observation that the maximal effect on glucose levels was achieved with $\geq 80\%$ inhibition of the type II diabetes target dipeptidyl peptidase-4 (DPP-4),^{60,61} and the demonstration by PET imaging that 80% occupancy of the serotonin (5-HT) transporter (5-HTT) was required for antidepressant efficacy of SSRIs,⁶³ and >90% occupancy of the neurokinin-1 (NK₁) receptor was required for the antiemetic activity of aprepitant.⁶²

■ DRUG–TARGET KINETICS AND CNS TUMORS

As discussed in this review, the application of drug–target kinetics to different disease states, such as in neurooncology, requires explicit knowledge of both the thermodynamics and kinetics of drug binding. In this regard, kinase inhibitor discovery programs provide a fertile ground on which to test and implement approaches based on kinetic selectivity for several reasons. First, a number of well-characterized kinases are targets for treating CNS tumors,² and access to kinase panels enables binding to potential off-target kinases to be assessed. In addition, kinase enzymology has been heavily studied, and thus in many cases there is a detailed understanding of mechanisms that lead to long residence time inhibition, such as binding to the DFG-out conformation or the development of covalent inhibitors.^{33,64,65} Finally, recognition of the potential importance of kinetic selectivity has stimulated the interrogation of structure kinetic relationships for both purified kinases and for kinase inhibitors in cell-based assays.^{66–68} For example, as noted above, although lapatinib binds ~10-fold less potently to EGFR than gefitinib (K_i^{app} values of 3 and 0.4 nM, respectively), the residence time of lapatinib on EGFR is >30-fold longer (430 and <14 min). The increase in lapatinib residence time was shown to translate to prolonged biochemical activity in a cell washout experiment (15% recovery 96 h after washout).³⁴ In addition, drug–target kinetics have also been employed in an attempt to account for the reduced efficacy of erlotinib for treating glioblastoma based on the observation that the residence time of erlotinib on the EGFRvIII mutant found in gliomas is shorter than that for the L858R EGFR mutant associated with NSCLC.⁶⁹

As mentioned at the beginning of this review, neurooncology drug discovery faces the additional hurdle of developing agents to penetrate the BBB. A recent review provides an authoritative summary of kinase inhibitors in this disease space and notes only a few examples of compounds that have been explicitly developed to be brain penetrant, such as inhibitors of EGFR and PI3K/mTOR.² For example, in contrast to the classic NSCLC EGFR inhibitors,⁷⁰ the irreversible pan-ERBB inhibitor NT113 has improved biodistribution to the CNS and has efficacy in intracranial glioblastoma xenografts, including those with high EGFRvIII expression.⁷¹ In addition, AZD3759 and GDC-0084 are reversible inhibitors of EGFR and PI3K/mTOR, respectively, both of which were designed to cross the BBB and show efficacy in preclinical models.^{72,73} However, other than the knowledge that NT113 is an irreversible inhibitor (which may contribute to improved efficacy of this compound compared to the classic reversible EGFR inhibitors), detailed kinetic studies on target binding and data from cell washout experiments are not available for these inhibitors limiting the conclusions that can be drawn about the relationship between target occupancy and efficacy.

■ SUMMARY AND FUTURE DIRECTIONS

The above discussion thus mitigates integrated efforts to generate time-dependent inhibitors of targets and evaluate the role played by parameters such as residence time in cellular washout experiments, and in more complex biological systems such as preclinical disease models. These efforts must be linked to mathematical approaches, such as PK/PD modeling, that link target engagement, drug concentration, and effect, and will be enhanced by direct measurements of target turnover, target levels, drug concentration, and target engagement. While the

determination of the kinetic parameters for enzyme inhibition and receptor interactions is de rigueur for enzymologists and quantitative biochemists, and slow-binding inhibition has been recognized for more than 40 years (see Morrison and Walsh,³ and references therein), it is perhaps curious that drug–target kinetics and concepts such as kinetic selectivity are not more widely used in drug development. One reason might be that the time-dependent assays required to measure residence time are intrinsically more challenging than the straightforward dose–response relationships (IC_{50} values) that are derived from the end point assays used in high throughput automated compound screens and that dominate biological structure–activity relationship. Moving forward, the implementation of programs that utilize drug–target kinetics will involve the identification of compounds that display time-dependent binding and the subsequent development of structure–kinetic relationships (SKR) for target binding through combined med chem/kinetic efforts. This, coupled with structure-based design and methods to analyze and simulate protein dynamics,^{15,74,75} will guide the synthesis of compounds with optimized binding parameters. In turn, compound series that encompass a range of binding kinetics can then be used to explore kinetic selectivity in cells and preclinical models. We note that some CROs now offer services to generate drug–target kinetic data, and techniques such as surface plasmon resonance (SPR) are routinely used to determine binding kinetics for purified targets, supplementing approaches such as forward and reverse progress curve assays. In addition, inhibition studies often include “ IC_{50} -shift” measurements in which a reduction in IC_{50} following preincubation of enzyme and inhibitor is taken as evidence for slow-onset inhibition (at least for a competitive inhibitor). Future advances will involve the routine implementation of methods to measure binding kinetics in the cell, such as those derived from NanoBRET,⁷⁶ approaches to quantify in vivo target engagement by reversible inhibitors,⁷⁷ and the further development and parametrization of advanced mathematical models that better simulate and predict drug–target interactions in the complex environment of the human body.^{21,22,24,26}

In summary, the availability of time-dependent target engagement data, both on purified targets and from cell washout experiments, will provide an additional dimension of information when selecting and optimizing drug leads including those that target brain cancers. In particular, prolonged target occupancy has the potential to translate into extended pharmacological activity at low drug concentration, which may be particularly important in neurooncology where drug exposure will be impacted by the BBB. However, the reliance on IC_{50} (or K_i , K_d) values for selecting and optimizing drug leads limits the ability to identify time-dependent inhibitors and ignores the possibility that kinetic selectivity may be present and could contribute to improvements in therapeutic window and safety.

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