Commitment to covalency: kinetics of irreversible inhibitors with a regenerable streptavidin sensor on the Pioneer FE system

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Introduction

The principal role of assay groups in drug discovery is to provide reliable methods, analysis, and data for confident decision-making about series progression. Particular assays are chosen to differentiate between affinity, specificity, cellular action, and most importantly mechanism of action. Increasingly, drug discovery faces challenges from harder-to-drug targets along with regulatory pressures to increase safety, efficacy, and to improve drug ADME (absorption, distribution, metabolism, excretion) properties. The responses to these challenges come through the creative use of both emergent and standard technologies, investing in new discovery approaches (e.g. fragments), and increasing exploration of drugs that act through targeted covalent modification.

Approximately 30% of the therapeutic enzyme inhibitors on the market function through covalent modification of the target. Concerns of specificity, metabolite reactivity, immunogenicity, clearance, redox activity, etc., often blunt interest in pursuing covalents as a primary discovery strategy. Contributing to the higher barrier to portfolio entry is a lesser familiarity with targeted covalent inhibitors and an underdeveloped biochemical toolkit for characterizing and describing the mechanism of action for this class of compounds.

Factors contributing to the potency of irreversible inhibition are poorly understood. An incorrect assumption often made is that covalent inhibitors don't require the intrinsic potency needed for non-covalent inhibitors since the reaction will make the binding permanent. Rather, the efficiency of the irreversible reaction relies on the specificity and potency of the initial encounter complex (binding). If the encounter complex does not form long enough for covalent bond formation to occur then chemistry can't assist. Therefore, the necessary work of making a potent, noncovalent binding scaffold is still critical. Similarly, understanding the covalent reaction in the context of the full drug and protein system is imperative since many factors can influence the rate and extent of reaction.

The simple two-step covalent inhibitor model (Equation 1) takes the same mathematical form as traditional Michaelis-Menten kinetics, and as such deriving an algebraic solution is non-trivial for highly optimized inhibitors.

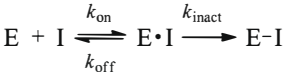


Figure 1: Rate equations for irreversible inhibition, where E is enzyme, I is inhibitor, E•I is encounter complex, and E-I is adduct or covalent complex. k_{on} and k_{off} are rate constants of association and dissociation, respectively. k_{inact} is the reaction rate constant of covalent inactivation.

The equilibrium approximation assumes that equilibration of diffusion controlled reactants to the final concentrations of substrate and product is instantaneous. Similarly, the quasi-steady state approximation assumes that intermediate complex forms much faster than the conversion to product. Neither of these assumptions are valid as the rate of covalent bond formation overtakes the rate of inhibitor dissociation; this is the goal of any successful covalent drug program. A more comprehensive model must be utilized and experimental methods to derive the constants developed. However, biochemical assays cannot unambiguously reveal the balance between binding and reactivity, further complicating structure-activity relationship (SAR) decision-making and additional information is necessary to resolve the contribution of each component.

Combining the results from multiple orthogonal techniques has proved particularly powerful in early discovery. In recent years, biophysical methods have made greater contributions both alone and in concert with other modalities. In this application note the work of Dr. Phillip Schwartz, Ph.D. of Takeda California, who champions covalent approaches in drug discovery, is highlighted. Through his recent papers and presentations, such as the 2016 Drug Discovery Conference discussed here, Dr. Schwartz combines the results from biochemical and biophysical experiments to provide a set of intuitive metrics for chemists to track as part of their SAR around covalent modifiers.

Analyzing data obtained from the Pioneer FE platform's implementation of Surface Plasmon Resonance (SPR) resolves inherent ambiguities in the biochemical data while providing additional parameters such as the direct observation of k_{on} and a metric for covalent reaction efficiency (C_c). SPR has enjoyed a growing role in drug discovery and provides direct observation of binding association and dissociation, in addition to quantitative kinetic parameters and a wealth of qualitative data discernable from curve shape. It can be used for fragment screening, mechanistic studies through competition assay formats, on-going program SAR, and assessing hits identified by orthogonal technologies — especially with activity assays for their direct binding components and stoichiometry.

Characterizing covalent binders with SPR is unusual given that the target protein is typically immobilized to the sensor chip and reused for different compounds and compound concentrations. This 'recycling' of the surface has generally targeted the technology at reversible systems and SPR labs often refrain from testing covalent modifiers over concerns of surface fouling. One method that avoids this concern is reversible affinity capture, which captures target and allows periodic replacement with fresh protein. However, affinity capture methods such as antibody or His-tag capture are often associated with drifting baselines which are detrimental for analyzing the kinetics of a covalent inhibitor. Capture of biotinylated target by immobilized streptavidin gives a desirable stable baseline, but regeneration of the surface for periodic replacement of target has proven inefficient as the biotin — streptavidin interaction is very high affinity and difficult to reverse. Specialized streptavidin reagents have been used with limited success but the reagents are costly and may not provide the necessary capture density to observe small molecule interactions. These issues can be overcome by capturing target-streptavidin complex onto a biotin sensor chip and regenerating with a harsh reagent which removes the protein content and leaves the surface ready for capture of fresh target protein. Experimental methods to stably capture target and analyze kinetics for irreversible inhibitors will be described.

This application note delineates Dr. Schwartz's method for combining a reversible streptavidin-biotin capture method with the real-time direct observation of binding/reaction using the Pioneer FE system with biochemical progress data to extract a full description of the inhibitor's protein association, dissociation, reactivity and their relative balance. This increased granularity should be invaluable to anyone pursuing covalent inhibitor SAR.

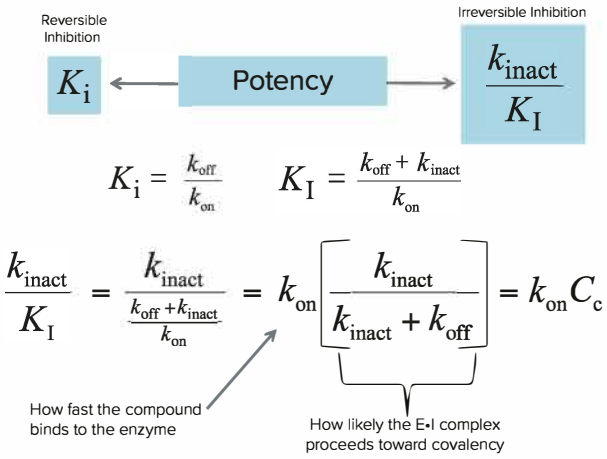


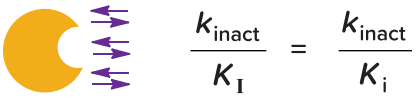
Figure 2: Equations derived by Dr. Schwartz to describe the kinetics of inactivation by covalent binders. K_i and K_I describe inhibitor potency for reversible and irreversible inhibition, respectively. k_{inact} is the rate of covalent reaction to form the adduct, E-I. By this model one sees that both affinity (K_i) for the target, as well as highly efficient chemistry (k_{inact}) are required to get efficient irreversible inhibition. The C_c term introduced is the commitment to covalency representing the probability that the encounter complex proceeds to form the adduct. The product of C_c and the association rate constant, k_{on}, describes the overall biochemical potency (k_{inact}/K_i).

Theory and results

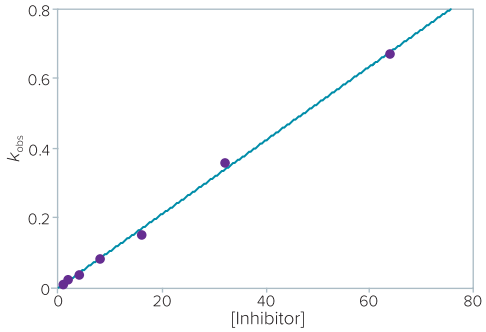
Dr. Schwartz's analytical method details contributions to an irreversible inhibitor's biochemical potency (k_{inact}/K_i), a parameter analogous to biochemical K_i, K_D or IC_{50}, that accounts for the extended covalent mechanism. Importantly, his model (Figure 2) allows for a compound to bind and partition to covalent bond formation with high efficiency. Traditional biochemical analysis relies on models that do not differentiate this type of highly efficient behavior, hence requiring abstraction beyond Michaelis-Menten-like kinetics. Introduced is the intuitive parameter commitment to covalency or C_c which relates to the efficiency of adduct (E-I) formation and its balance with dissociation. It is both the efficiency of adduct formation and the rate of protein/inhibitor association that defines biochemical potency.

Dr. Schwartz then demonstrates that this C_c parameter can be used as a diagnostic for the rate limiting step in the reaction. In Figure 3, he describes situations where: Case 1) neither binding nor reaction are efficient, Case 2) binding is more efficient, but the reaction is still not and compounds dissociate far more frequently than they react, Case 3) reaction is faster but binding is inefficient and Case 4) binding is potent and reaction is efficient. Unfortunately, the biochemical rate data (k_{obs}) alone look the same for Cases 1 and 4 or 2 and 3. There is still an ambiguity remaining, but SPR can be used to resolve it.

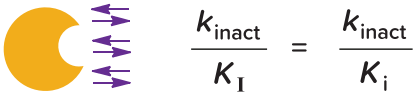
Case 1: k_{off} >> k_{inact} **Unbalanced**



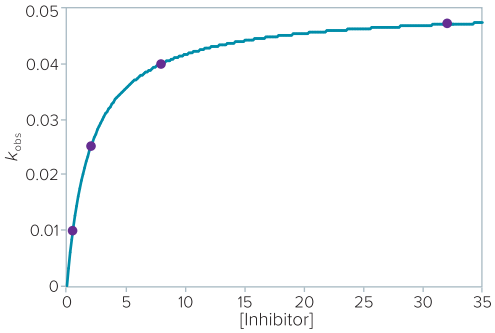
C_c << 0.1



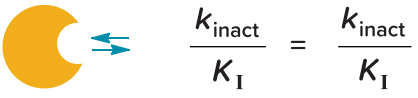
Case 2: k_{off} >> k_{inact} **Unbalanced**



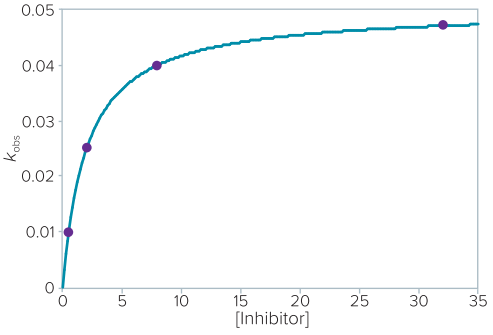
C_c < 0.1



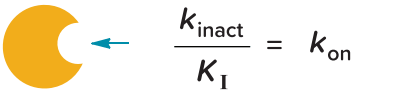
Case 3: k_{inact} = k_{off} **Near Balance**



0.1 < C_c < 0.6



Case 4: k_{inact} >> k_{off} **Balanced**



C_c > 0.6

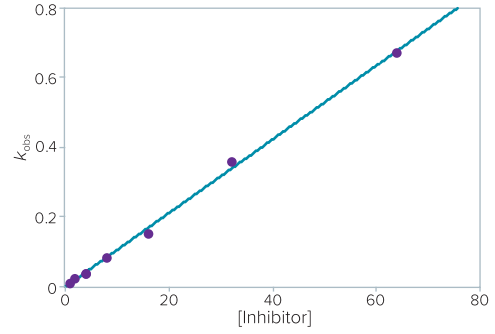


Figure 3: Examples of different Commitment to Covalency (C_c) cases and the observed results in enzyme assay (k_{obs}) data. Simulations show that initial rate assays cannot easily distinguish C_c contributions to covalent inhibitors.

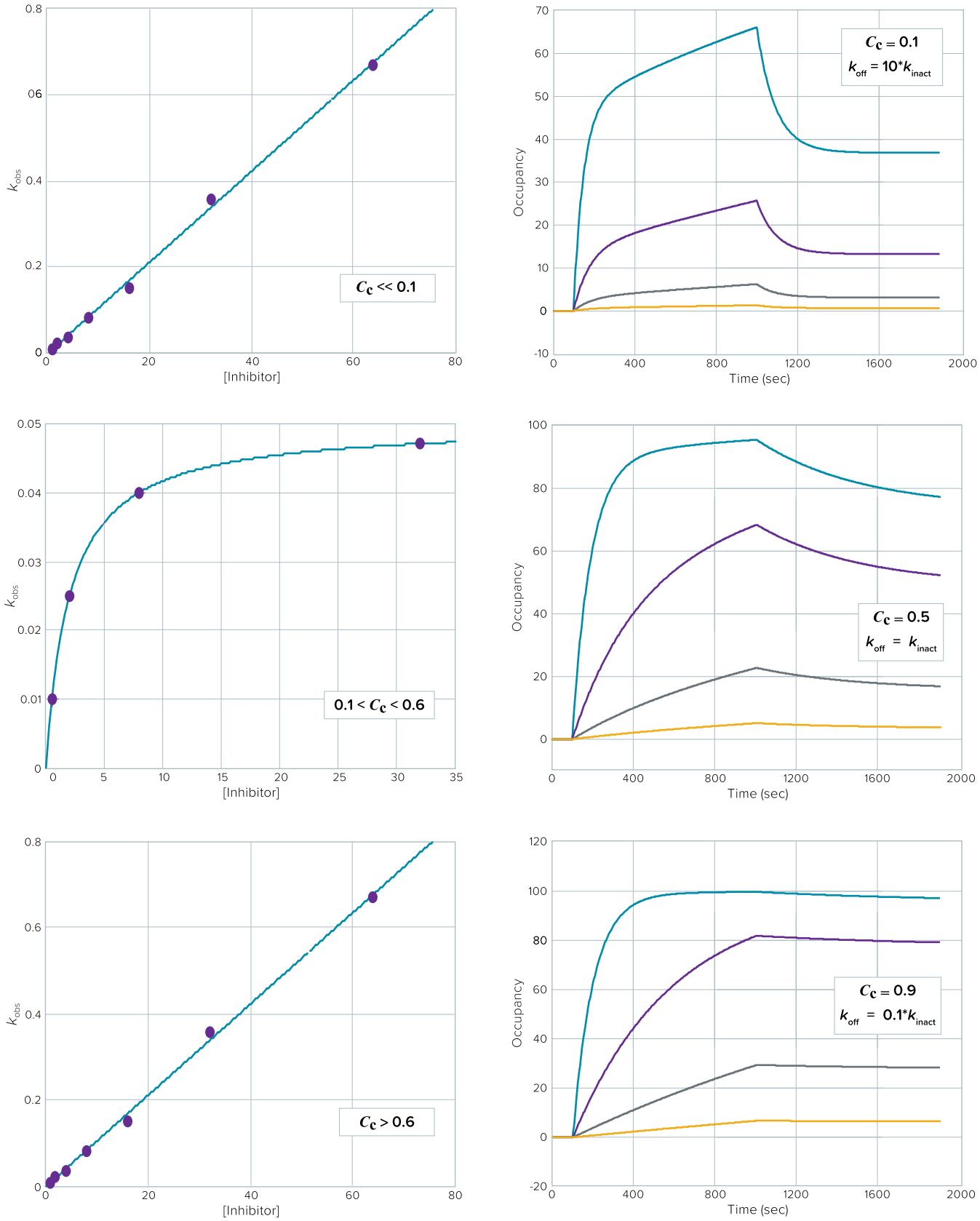


Figure 4: SPR can unmask C_c contributions. Enzyme assay simulation (left column) and SPR simulation (right column) of covalent compounds with differing C_c properties. Unreacted compound in these examples is shown to dissociate rapidly at the end of injection (1000 sec, top row) leaving reacted compound irreversibly bound. Compounds with higher C_c properties (middle and bottom rows) have less dissociation and more reacted compound. From the SPR responses one can unambiguously determine biochemical potency, rate of inhibitor association and the efficiency of the chemical reaction in the encounter complex.

The SPR response curves are evaluated for the 'distortion' to the pure binding/dissociation curves usually obtained for reversible inhibitors tested on Pioneer technology and shown in Figure 4. In the top row the reaction is slow relative to the dissociation (C_c << 0.1). At the end of the injection unreacted compound rapidly dissociates leaving behind only the small fraction that did react. If the off-rate and chemical rate (k_{inact}) are similar some dissociation is observed but a larger fraction is retained (middle row, 0.1 < C_c < 0.6). In the ideal case (Figure 4, bottom row, C_c > 0.6) the entire fraction that binds also reacts and no dissociation is observed. From a PK/PD perspective, a fully committed covalent inhibitor can have significant impact on dosage versus an inhibitor that must bind and release many times before target inactivation occurs.

The theory is put to practice using an improved affinity capture method which captures biotin-tagged target to reversibly immobilized streptavidin. The method takes advantage of the Pioneer FE system's robust fluidics by regenerating target-streptavidin complex with a harsh reagent. The benefits of this target capture method are:

- Improved target activity
- No requirement for specialized streptavidin reagents
- A reproducible capture yield for many test cycles

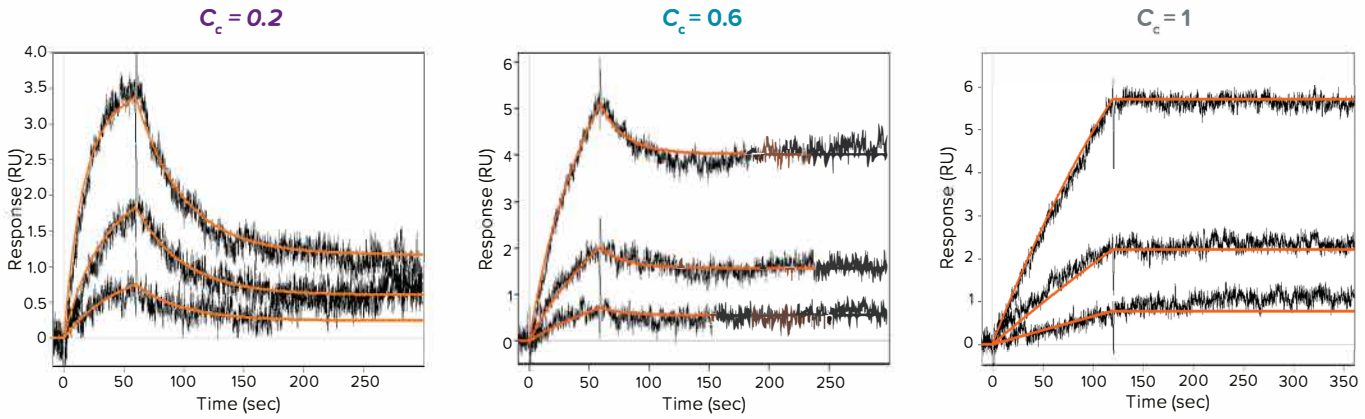
A biotin-tagged kinase target was pre-incubated with streptavidin and captured per the Desthiobiotin Sensor Capture Method outlined below. Binding data were fit with a form of the two-state kinetic model where k_{on}, k_{off}, and k_{inact} are determined at multiple analyte concentrations. C_c is also determined by the previously described relationship of k_{off} and k_{inact}. Three published covalent inhibitors were bound to target using this method and significantly different C_c values were observed for each.

For reference, an endpoint competition assay described before was used to independently determine k_{inact}/K_I. The ability for both assays to determine k_{inact}/K_I provides an internal consistency check while the SPR assay further distinguishes C_c from k_{on}.[1]

Experimental methods

DESTHIOBIOTIN SENSOR CAPTURE METHOD

1. Install a Pioneer COOHV or CDH biosensor and briefly condition with two injections each of 10 mM HCl, 50 mM NaOH, and 0.1% SDS.
2. Couple Desthiobiotin by injecting activation solution (0.2 M EDC + 0.05 M NHS) for 25 min at 10 µL/min, then injecting 2 mg/mL Amine-PEG_4-Desthiobiotin (Thermo Fisher Scientific Cat. # 16131 or other) in 50 mM HEPES pH 7.4 for 30 min at 5 µL/min, and deactivating by injecting 1M Ethanolamine HCl pH 8.5 for 5 min.
3. Pre-incubate monobiotinylated target protein with streptavidin in a 1:1 or 2:1 molar ratio to form target-streptavidin conjugate.
4. Capture target-streptavidin conjugate by injecting 25 µg/mL sample for 2–10 min at a flow rate of 10 µL/min, to desired capture density. Capture density may be improved by pre-



Published inhibitors	k_{on}	C_c	SPR k_{inact}/K_I ($M^{-1}s^{-1}$)	Biochemical assay k_{inact}/K_I ($M^{-1}s^{-1}$)
Kinase inhibitor 3	4.0E+05	0.2	7.8E+04	2.1E+05
Kinase inhibitor 2	1.0E+06	0.6	5.5E+05	7.3E+05
Kinase inhibitor 1	4.1E+05	1	4.1E+05	8.8E+05

Figure 5: SPR data from the Pioneer FE system showing three covalent inhibitors binding target with different C_c values. Signal after referencing shows stable baselines, suitable for measuring these potent inhibitors and differentiating binding from covalent reaction. Potency parameters are compared between inhibitors and assay methods to show the differences in reaction efficiency.

paring the conjugate in a preconcentration buffer approximately 1 pH unit below the conjugate isoelectric point (pI). For best baseline stability, the capture injection should stop before it reaches full saturation. The surface may require conditioning for cycle-to-cycle capture reproducibility, so include 4–6 capture-regeneration cycles to establish a reproducible capture signal prior to testing analytes.

5 Inject irreversible analyte sample using either a standard injection or a OneStep® Injection. For standard injections, use a 3–6 sample concentration series and inject each sample for 3–6 min at a flow rate between 25–75 µL/min. For OneStep Injections, inject three sample concentrations using the 100% sample loop volume setting at a flow rate between 25–75 µL/min. Include a 5–15 min dissociation in each injection cycle.

6 Regenerate the target-streptavidin conjugate by injecting 10 mg/mL iminobiotin dissolved in a 1:1 mixture of 200 mM NaOH with acetonitrile for 2 min.

7 Repeat Steps 3–6 for all analyte and reference (buffer) samples.

The Desthiobiotin Sensor method was discovered by the team at Takeda and this method enables an optional stepwise capture of streptavidin followed by biotinylated target. This method gives the desired result of stable capture of protein targets, is compatible with many buffer components and is fully regenerable. Data were referenced using reference channel response, and buffer blank signal and kinetics were fit with the two-state irreversible kinetic model (rate equation shown in Figure 1). C_c was calculated as shown in Figure 2.

Conclusion

The presented work reveals a useful extension of the mechanism of covalent binding drugs and provides a practical framework for significant differentiation between molecules. This proposed method represents an elegant blend of biochemical and biophysical methodologies and utilizes SPR technology in an innovative way to directly acquire the necessary data (k_{on}, k_{off}, k_{inact}) to resolve the ambiguity that the k_{obs} data alone cannot recover. This 'best of both worlds' strategy epitomizes the benefits gained from combining orthogonal approaches.

The parameter set described (k_{on} and C_c) is the reasonable minimal set of SAR terms that provide a comprehensive description of the inhibition mechanism. The Michaelis-Menten-like formulation of inhibitor action threatens to get unwieldy when the steady-state approximations are removed. The approach described here simplifies the additional terms into the C_c term, making the interpretation more broadly accessible. C_c may be to covalent inhibitors what net rate constants have been to mechanistic enzymology since the last century.

Looking forward, it is exciting to imagine the use of the refined gradient injection mode offered by the Pioneer instruments (OneStep Injections) to provide even more precise estimates of the association-phase information, and underlying off-rate where labeling is incomplete or there is intrinsic reversibility. OneStep Injections provide for more kinetic curvature in the binding association, and in the irreversible case the inflection point of the association may provide additional meaningful internal consistency checks of the reaction data. Additionally, new target capture methods create opportunity for the analysis of covalent inhibitors against more targets and target classes. Kudos to Dr. Schwartz and the team at Takeda for advancing the state of the art for this kind of drug discovery.

Acknowledgment

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References

I Miyahisa, T Sameshima, MS Hixon, Rapid determination of the Specificity Constant of Irreversible Inhibitors (k_{inact}/K_I) by Means of an Endpoint Competition Assay, *Angew Chem Int Ed Engl*, 54(47): 14099–14102, 2015.