

Identification and characterization of binding interactions of aggregating or promiscuous small molecules

Abstract

Small drug-like compounds that do not fit the classical 1:1 binding inhibition behavior have been described and potentially act through formation of compound aggregates. Determining whether these aggregates cause promiscuous inhibition or more desirable target-specific inhibition is critical for prioritizing compound progression through the drug development pipeline. SRU Biosystems' BIND[®] platform is a label-free detection technology used for biochemical and cell-based drug discovery applications. BIND technology produces highly quantitative data with kinetic readout and is uniquely positioned for characterizing the mechanism of action of small molecule inhibitors. This application note discusses experimental set up and data interpretation of high throughput label-free BIND assays designed to address the time-course, stoichiometry and specificity of compound binding.

Introduction

Recent articles [1-6] have described issues associated with small drug-like compounds (<600Da) that do not fit the classical 1:1 stoichiometric target association model. Particular attention has focused on a behavior that is associated with formation of small compound aggregates (size 30-400 nm) that can interact with protein surfaces and thereby inactivate targets. Some compounds displaying this behavior inhibit a wide range of different proteins, and hence have been termed “promiscuous”, though other compounds can show remarkable specificity and potency. Such behavior can lead to the initial progression of compounds with undesirable properties or conversely result in the omission of weaker but more desirable binders. Measurements based on effects of function have been used to distinguish such behavior from classical inhibitors [1-4], and also aggregation itself can be utilized [3,5].

Label-free microplate-based BIND[®] Reader and Biosensors offer a simple and more generic way to identify compounds that might work by this undesirable function. BIND[®] enables efficient prioritization of those compounds that are best behaved and likely to be authentic hits. Label-free detection methods by their very nature can detect all binding events both specific and non-specific. BIND technology users should have heightened awareness to the size of PWV shifts,

BIND time course behaviors and their most appropriate interpretations, in order to best characterize the binding of small drug-like compounds in support of screening and profiling applications. This technical note provides guidance and suggestions on how to characterize the binding interactions of hits coming out of your screening programs.

BIND[®] Technology

BIND is a plate-based biosensor system capable of highly quantitative binding analyses. The PWV (Peak Wavelength Value) shift signal (nm) obtained when a molecule binds directly to the sensor or to an immobilized target, is directly proportional to the mass of the molecule bound. The

Figure 1

maximum PWV shift expected from the binding of a ligand to its immobilized target can be calculated using the formula:

$$\text{ligand PWV}_{\text{expected}} = (\text{MW ligand} / \text{MW target}) \times (\text{PWV target}) \times n \times z \times \% \text{ target activity}$$

with n = number of binding site per molecule of target and z = % of the immobilized target that is functional for ligand binding.

Generally, well-behaved compounds will bind to a target with a measured stoichiometry of <1:1. The stoichiometry would ideally be normalized to that seen at saturation with a standard binder to account for the level of functional activity of the target. Higher apparent stoichiometries suggest an element of non-specific binding.

For most targets with K_d s in the range 0.1->1000 μM , the association rate constant for a drug-like small molecule binding to a target will be upwards of $10^6 \text{ M}^{-1}\text{s}^{-1}$, and often 10^7 or higher. At $10^6 \text{ M}^{-1}\text{s}^{-1}$, with 10 μM compound, the on-rate would be 10 s^{-1} , corresponding to a half-time of association of just 60 msec. Thus, binding is complete within 1 sec, and well before the first read after addition. Any slower binding is indicative of either non-specific binding, or a rate-determining structural change in the target or ligand.

Single Concentration Testing

Information from time-courses and stoichiometry

A typical BIND primary high throughput screen (HTS) would test small molecule compounds at 0.1-2 % DMSO and 10 μM compound concentration. A similar protocol might be used as an orthogonal screen for secondary characterization

of hits from an uHTS campaigns that have a high rate of actives (e.g. false positives from fluorescence interference).

BIND users will have a target-coated biosensor microplate equilibrated in assay buffer and perform the following steps:

1. Record a baseline for 1-5 minutes to determine sensor starting PWV and stability
2. Remove the sensor microplate to a liquid handling platform for uniform, simultaneous addition and mixing of compounds to all the wells on the sensor
3. Return the sensor microplate to the instrument and record a time-course (~ 4 minutes) of the binding interaction

These steps are repeated until all of the sensor microplates have been processed. All label-free screening methods are subject to DMSO mismatch contributions from the compound library. DMSO mismatches can be minimized by utilizing the standard BIND assay optimization protocol in which the DMSO final concentration is as low as possible (<1 %).

Table 1 details categories of expected responses for 10 μM single concentration screening using label-free microplate-based BIND Biosensors. The most well behaved compound type is shown at the top of the table and the least likely to be a true hit is shown as the last entry in the table.

Compound Secondary Analysis

Analysis of dose-response curves

Generally, well-behaved compounds with K_d s in the range 0.5->1000 μM , will give a dose-response curve that shows

Apparent Stoichiometry	Information from time-course	Most Likely Interpretations	Recommendations
$\leq 1:1$	Complete response within mixing time (see D in Figure 1)	a) specific binder b) non-specific binder assayed at non-saturating concentration	a) or b): Perform dose-response to determine K_d , stoichiometry of specific binding. This also identifies any non-specific component.
$\leq 1:1$	Slow monophasic response from zero to <1:1 (not shown in Figure 1)	Slow-binding, mechanistically interesting compound	Consider that binding requires the target to undergo a conformation change Perform a dose-response curve, with sufficiently long time-courses to achieve equilibrium to confirm 1:1 specific, but slow, binding
Between 1:1 & 5:1	Either, complete response within mixing time (see D in Figure 1) Or slow response, often without achievement of an equilibrium level (see C in Figure 1)	a) specific binder, combined with a non-specific component b) slow-binding, aggregating compound c) Potent binder, with significant non-specific binding, when assayed at $\gg K_d$	a) or b) Caution that compound might be mechanistically-unacceptable in BIND and other biochemical assays Perform dose-response to look for a specific binding component. c): Identification from shape of dose-response curve using appropriate concentration range
>5:1	Rapid or slow binding (see A in Figure 1)	Most likely binding of aggregated compound	Compound might show an undesirable MOA in other biochemical assays. BIND would not be able to detect any specific component.

Table 1: Categories of responses commonly seen during a single concentration screen

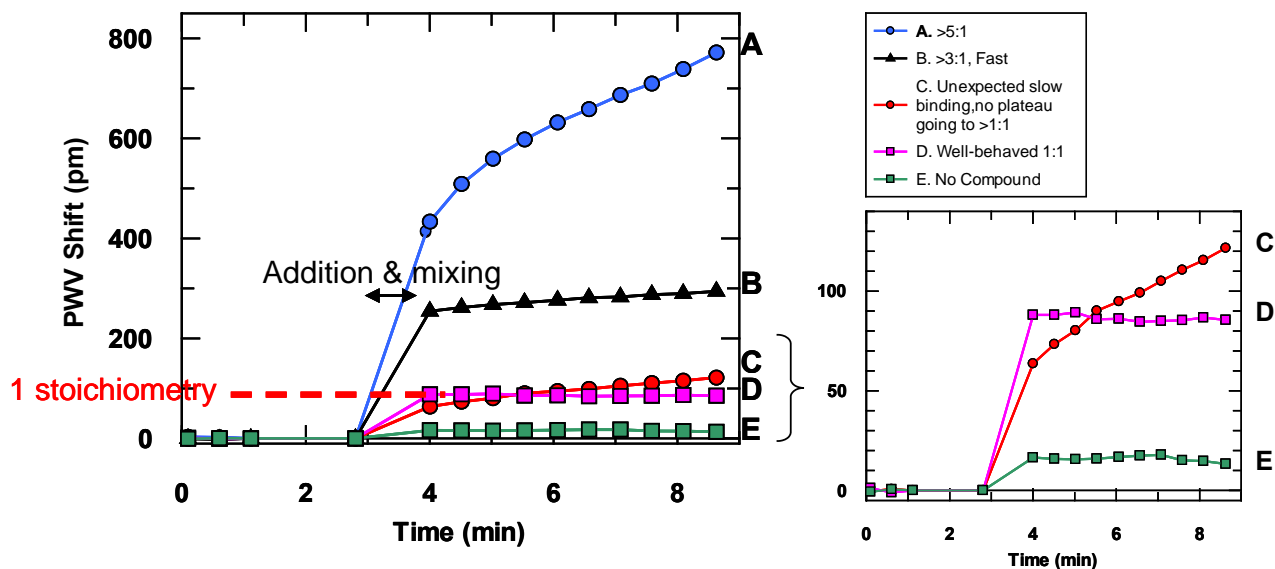


Figure 1: Mechanistic insight from timecourses of binding.

This figure shows examples of BIND time-courses with mechanistically acceptable binding profiles and others that have varying levels of non-specific interaction. The plots shown represent examples of each category of compound binding (A,B,C,D) response commonly seen during a single concentration screen (see Table 1). The insert provides a closer look at the binding timecourses for compounds in category C and D.

saturation with a stoichiometry consistent with 1 molecule of ligand bound per binding site and a K_d compatible with solution measurement.

With industry-standard 96- and 384-well BIND Biosensor plates, dose-responses can be readily obtained in high throughput and the resultant data fitted to appropriate 1:1 binding molecules to derive K_d and stoichiometry. Moreover, appropriate models can be utilized which allow the discrimination of a saturable specific binding component from any non-specific responses (Figure 2). Such data can generally be obtained by titrations on a target-surface and do not necessitate reference or control surfaces.

It is recommended that dose-response curves are performed on all possible hits, because this will greatly increase the confidence in discriminating specific, from non-specific responses, as well as giving highly quantitative affinity data for specific binders.

Information on hits from competition experiments

When a standard binding compound that occupies a specific site is available, or is discovered during the screening process, BIND provides a very effective means of distinguishing

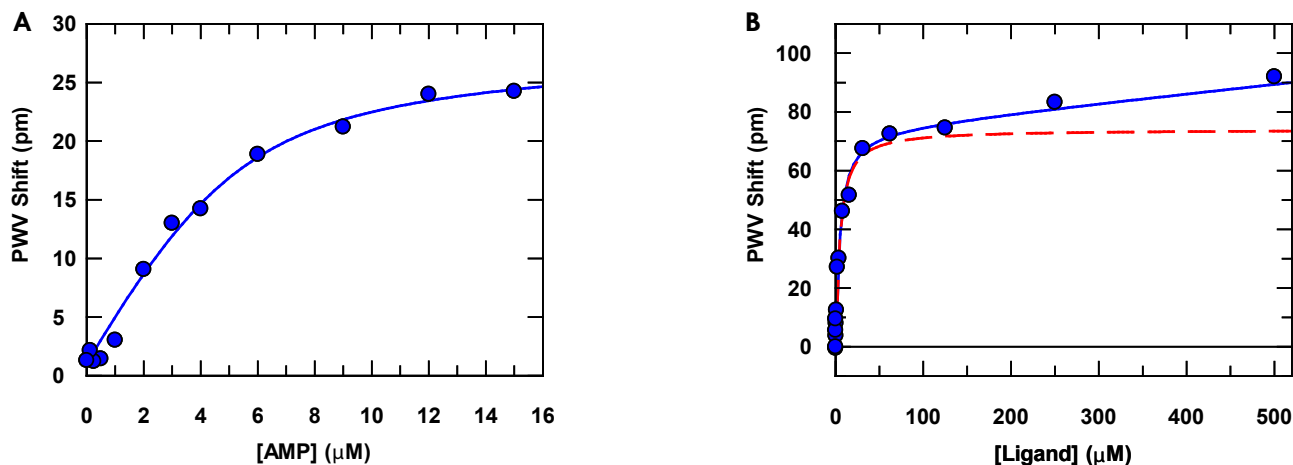


Figure 2: Use of dose-response curves to give K_d , stoichiometry and detect non-specific binding.

A Example of a compound which shows only specific, saturable binding. The solid line is the fit to a 1:1 binding model with a K_d of 1.2 μM and a stoichiometry close to 1:1 (1:1 stoichiometry is 26 pm). B Example of a compound which binds specifically at low concentrations around its K_d but shows additional non-specific binding at and above its K_d . The full-dose response curve does not show saturation and goes above 1:1 stoichiometry (1:1 stoichiometry is about 70 pm). The solid line shows how such data can readily be fitted to a model based on 1:1 binding combined with a linear function to represent a non-specific component to yield the K_d (4 μM) and stoichiometry of the specific binding component. The dashed line shows the predicted binding curve for specific binding with 1:1 stoichiometry.

specific site binders from any non-specific responses. Experimentally, pairs of target-coated sensor microplates are used. To one the known site binder is added, at sufficient concentration as to prevent binding by test compounds at that site. The set of test compounds is then added to both plates. The difference in response between the two plates to the test compounds is a direct measurement of specific-site binding (see Figure 3)

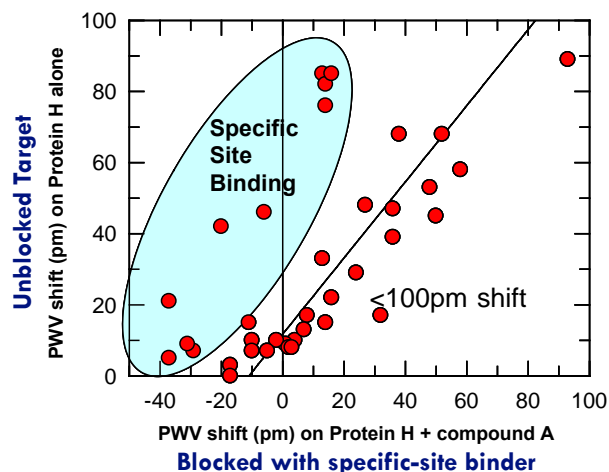


Figure 3: Confirmation of specific-site binding by competition

The ATPase domain target was immobilized in all biosensor wells and saturating amounts of a reversible nM specific site-binder was added to half of the plate. Test compounds were added in duplicate to each half of the plate and binding of test compounds to the target (blocked or unblocked) was measured on BIND. A plot of the signal on blocked vs. unblocked is useful to determine same site binding.

Suggested ways to help limit aggregation & insolubility issues

1. Compound aggregation is concentration-dependent: Assay at lowest compound concentration consistent with ability to detect binding
2. Compound aggregation is dependent on the protocol for dilution from DMSO into aqueous buffer: Make a direct single dilution from stock in DMSO into the final DMSO concentration and screen as soon thereafter as possible.
3. Behaviour of Compound aggregates can be affected by additives: Include small amounts of the detergents (e.g. 0.01% v/v Tween-20) or BSA (<math><10\ \mu\text{M}</math>) in the final binding buffer
4. Compounds that are known to precipitate at the concentrations tested should be centrifuged or filtered and retested on BIND to confirm binding mechanism.

Advantages of BIND[®]

- Equilibrium binding measurements quantify stoichiometry and affinity
- Short time-courses give extra information in distinguishing specific from non-specific hits
- Identification of mechanistically unacceptable compounds both in a primary screening mode and as an orthogonal secondary screen.
- Progression of single concentration hits via dose-response curves and competition experiments increases confidence in the quality and specificity of hits.
- The BIND technology has significant advantages over flow-SPR, and other label-free methods
 - A direct assay in a range of standard microplate formats (96-, 384- and 1536-well) gives very high throughput (up to 10₆ data points/ 8 hour day) with a simple to use, automation friendly reader
 - A single result from a single well avoids the complications sometimes seen with time or reagent denatured targets and regenerated surfaces
 - Multiple dose response curves are obtained quickly and simultaneously giving excellent quality control and high throughput

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