

Surface Plasmon Resonance Based Assay for the Detection and Characterization of Promiscuous Inhibitors

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Promiscuous binders achieve enzyme inhibition using a nonspecific aggregation-type binding mechanism to proteins. These compounds are a source of false-positive hits in biochemical inhibition assays and should be removed from screening hit lists because they are not good candidates to initiate medicinal chemistry programs. We introduce a robust approach to identify these molecules early in the lead generation process using real time surface plasmon resonance based biosensors to observe the behavior of the binding interactions between promiscuous compounds and proteins. Furthermore, the time resolution of the assay reveals a number of distinct mechanisms that promiscuous compounds employ to inhibit enzyme function and indicate that the type of mechanism can vary depending on the protein target. A classification scheme for these compounds is presented that can be used to rapidly characterize the hits from high-throughput screens and eliminate compounds with a nonspecific mechanism of inhibition.

Introduction

A key step in the drug discovery process is the generation of lead compounds that can be developed into potent inhibitors. One approach to lead generation is the screening of chemical compound libraries against a protein of interest to identify hits that can be further developed into a lead series.¹ An essential part of any screening process is the identification and elimination of false positive hits. Some false positives in high-throughput biochemical screens result from compounds that can covalently react with the protein or possess spectral properties that interfere with fluorescent or colorimetric readouts.² Another more recently described class of false positives termed promiscuous binders are characterized by their ability to inhibit a broad spectrum of different protein classes, often potently, obscuring well behaved, but weaker binding, desirable hits.^{3,4} Developing techniques to identify and eliminate promiscuous binders is crucial to the goal of providing high-quality candidate compounds for lead generation activities. In addition, these same assays can yield mechanistic understanding of how promiscuous binders achieve protein inhibition.

Investigations of promiscuously binding compounds indicate that in solution they often form soluble or colloidal aggregates ~30–400 nm in diameter in solution.³ Imaging studies show that these aggregates, which can bind to proteins with high affinity, envelop the protein preventing substrate access and inhibiting the protein's function.^{3,4} This is a nonspecific mechanism of inhibition and cannot be further developed by medicinal chemistry to produce viable drugs. Biochemical studies show that very often the potency of these compounds changes with time,³ protein concentration,³ and presence of detergent^{4,5} and can exhibit dose response curves with extremely steep Hill slopes due to their potent binding properties.⁶ Several screening approaches to identify promiscuous compounds early in the discovery process have been proposed. Examples include static or dynamic light scattering of compound solutions to identify aggregators;⁷ characterizing hits in the screening assay with the addition of detergent⁸ or high concentrations of BSA⁹

and eliminating compounds with a marked reduction in potency in the presence of the additives; examination of time-dependent inhibition;⁶ and rescreening hits at different protein concentrations looking for protein concentration-dependent IC₅₀ values.⁶ Light scattering is a commonly implemented method and has even been applied to a large scale screen of about 70 000 compounds.¹⁰

We sought to expand our understanding of promiscuous inhibitors by studying their binding properties to proteins in real time using a surface plasmon resonance (SPR)¹¹ based binding assay as implemented with Biacore technology. Dissolved compounds are injected over protein surfaces attached to an optical biosensor surface (association phase), and binding is read out in real time as the change in mass at the sensor surface.^{11,12} After the injection, running buffer is flowed over the surface and dissociation of the compound from the surface is observed. This assay allowed us to assess how these compounds associate and dissociate from proteins in real time, giving a more detailed view of how the compounds interact with proteins.

We selected 13 known promiscuous compounds (Figure 1 and methods) and characterized their binding to seven proteins and a blank surface in the presence and absence of detergent. A majority of the test compounds bound to most or all of the target proteins, an expected feature of promiscuous inhibitors. We observed that most compounds bind at stoichiometric ratios greater than 1:1 with a large range of stoichiometries. Some compound binding was found to be fully reversible, despite binding at high superstoichiometric ratios. Examination of all interactions in the presence and absence of detergent revealed that many, but not all, interactions were detergent sensitive. We also found that some compounds behaved well at low concentration but exhibited nonstoichiometric binding at higher concentrations. Surprisingly, we found that there are different

*Abbreviations: SPR, surface plasmon resonance; RU, resonance unit; R_{max}, maximum binding response at surface saturation; IRAK4, interleukin-1 receptor-associated kinase 4; JNK2, c-Jun kinase 2; p38, mitogen activated protein kinase 14; HIV-RT, human immunodeficiency virus 1 reverse transcriptase; HCVpol, hepatitis C virus polymerase; HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; CMC, critical micelle concentration

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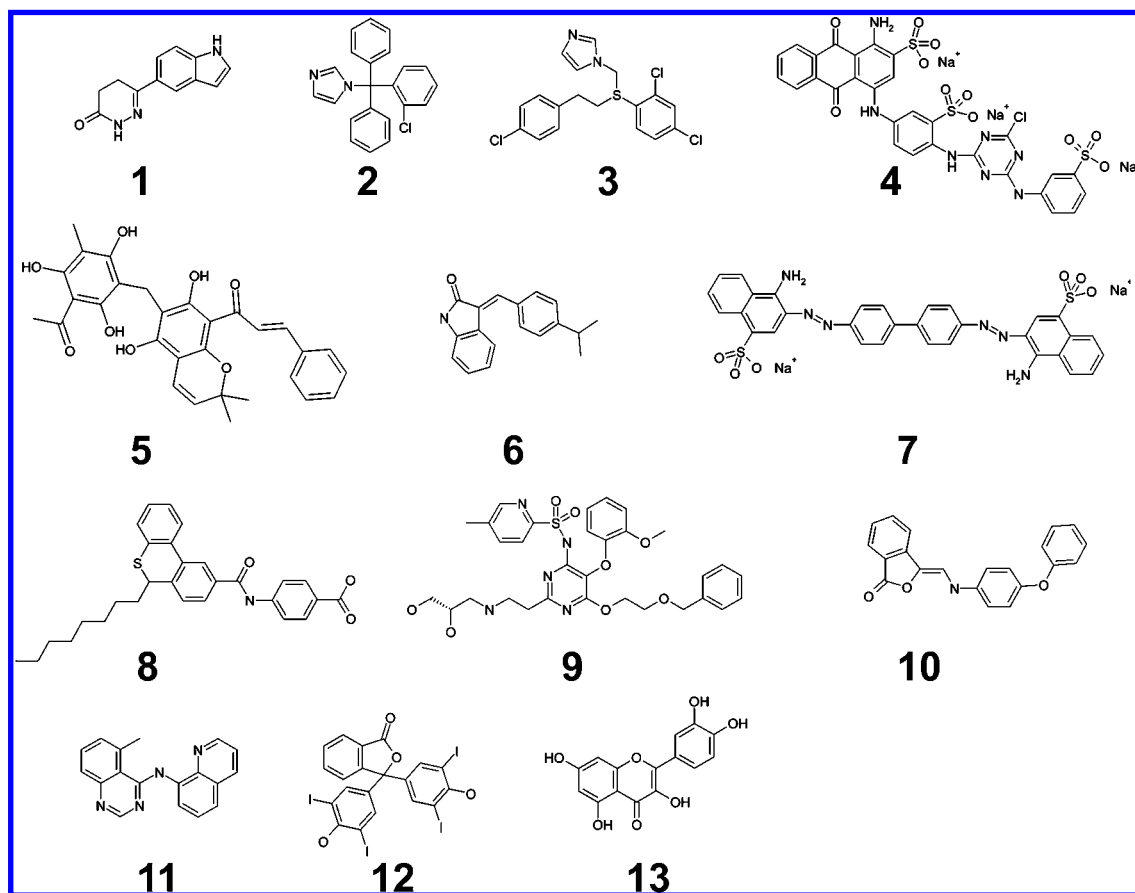


Figure 1. Chemical structures of known promiscuous binders chosen for this study. Common names for some compounds are as follows: **2**, clotrimazole; **3**, sulconazole; **4**, cibacron blue 3ga; **5**, rottlerin; **6**, MFCD00118155; **7**, congo red; **10**, MFCD00139657; **12**, tetraiodophenylphthalien; **13**, quercetin.

degrees of promiscuity in binding. Some compound aggregates interacted with some proteins and not others, and the mechanism of interaction varied from protein to protein. We present a classification scheme for the binding behavior exhibited by promiscuous binders. Furthermore, we demonstrate that SPR can be used as a rapid secondary assay to identify promiscuous binders and eliminate this source of false-positive hits identified by enzymatic screening.

Results

Well-Behaved Compounds. Before characterizing the known promiscuously binding compounds (Figure 1), we demonstrated a working assay for each of the proteins utilized in this study. Binding experiments with control compounds (sensorgrams in Figure 2 and Supporting Information Figure 1) exhibited the kind of behavior desired in a compound at the hit-to-lead stage of lead identification.² They showed concentration-dependent binding and reversibility, eventually dissociating to baseline. The data were well described by a 1:1 binding interaction model (Figure 2 and Supporting Information Figure 1). The compounds were also reasonably selective and showed little to no interaction with other proteins (data not shown). While strict specificity is not necessarily required early in a lead discovery program, a good lead should not be a highly potent inhibitor of many other proteins. These binding profiles contrast sharply with the behavior of known promiscuous compounds (e.g. Figure 3A) that have a variety of nonideal behaviors as described below.

Assessing Binding Stoichiometries. Nonstoichiometric binding behavior is characterized by binding responses that significantly exceed the maximal expected binding level (R_{\max}) to a

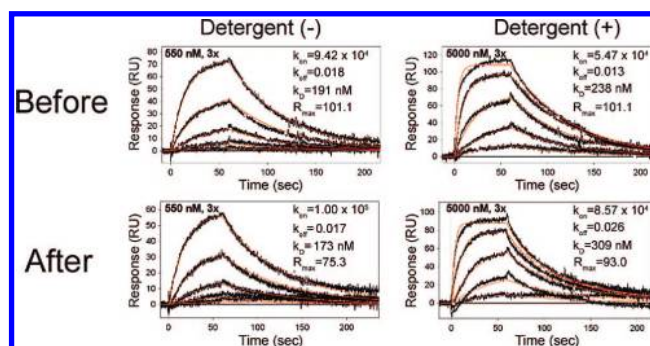


Figure 2. Four independent experiments of control compound binding to HCVpol demonstrating the desirable properties of compound/protein binding. Binding is saturable, fully reversible, and agrees well with a 1:1 binding model (red lines). Top panels show the behavior of freshly prepared HCVpol surfaces with or without 0.005% Tween-20 detergent in the buffer. Bottom panels show the behavior of the samples after 20 h of exposure to various concentrations of 13 known promiscuously binding compounds. There are no significant changes to the on- or off-rates or the K_D . The ~25% loss in binding capacity in the detergent-free experiment is reduced to a negligible 7% loss in the detergent-added experiment. Each figure is labeled with the highest concentration injection shown and the dilution factor relating subsequent injections.

protein surface, estimated by comparison to saturating concentrations of a control compound (see methods section). Compounds exceeding 5 times the R_{\max} are identified as superstoichiometric binders. Some achieved upwards of 140-fold the maximal binding in a 1 min association phase (Figure 3C, HIV-RT) and showed no indication of achieving saturation or

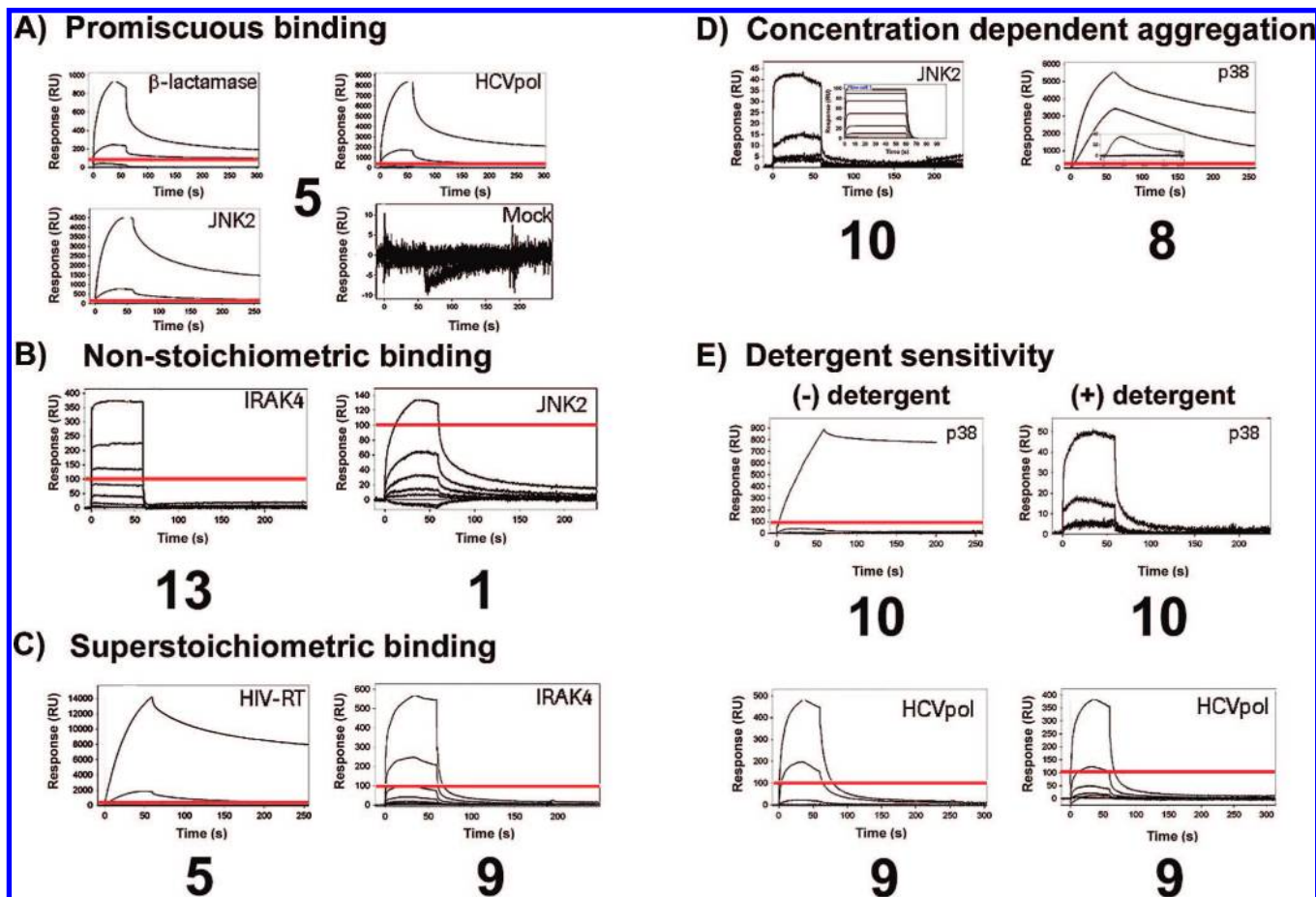


Figure 3. Classes of nonideal binding behavior. Red lines indicated 100% surface saturation for a 1:1 compound/protein interaction (see methods section). Additional examples of each behavior are available in the Supporting Information figure and table. (A) Compound **5** binds at superstoichiometric ratios to all protein surfaces ($R_{\max} = 100$ RU). (B) Lack of apparent saturation near the R_{\max} (red lines) is indicative of nonstoichiometric binding. (C) Examples of superstoichiometric interactions are shown. Both reversible (IRAK4) and irreversible (HCVpol) examples are given. Note the lack of any apparent approach to equilibrium for the HIV-RT example. (D) Examples of concentration-dependent aggregation are shown. Note that the top concentration has a significantly different response level or shape profile compared with the lower concentrations. In the case of p38 binding compound **8**, the inset shows the lowest five concentrations in the dilution series. An increase in concentration of only 3-fold changes the behavior from no signal (lowest four concentrations) to ~ 30 RU of observed binding. An additional 3-fold increase in concentrations results in ~ 3000 RU of binding and again to ~ 6000 . For **10** and JNK2, the responses have not exceeded R_{\max} but the increase in response is significantly disproportionate from that expected for 1:1 binding (inset). (E) Some interactions show a marked changed in behavior upon addition of detergent (p38 binding **10**), while others do not (HCVpol binding **9**).

equilibrium. In one experiment, compound **1** was allowed to associate for more than 12 h (by including it in the running buffer) and was still accumulating on the chip surface at the end of the experiment (data not shown). Since these data are reference subtracted, this result was not due to nonspecific interactions with the sensor surface but instead resulted from the compound's interaction with the protein. SPR cannot unambiguously discern the mechanism for this kind of surface buildup. However, light scattering, confocal microscopy, and electron microscopy results indicate that these compounds form large aggregated structures in solution that envelop the protein.⁴ This suggests that the large signal observed in the SPR experiment results from the interaction of a massive aggregate of compound that has some intrinsic binding affinity for the protein. That many of the tested interactions do not achieve equilibrium could result from continued recruitment of monomers to the aggregate or from a slow approach to equilibrium due to slow binding kinetics and low concentration, as might be expected for the diffusion of a large aggregate. For example, a 50 μM solution of compound monomers aggregated into 30 nm particles, as observed in EM experiments,³ would yield a 1.1 nM solution of aggregates.

Superstoichiometric binders are not good candidates for additional lead-generation activities. In contrast, compounds that exceeded R_{\max} by less than 5-fold were labeled as nonstoichiometric binders (Figure 3B). There are several reasons a well behaved molecule may appear nonstoichiometric. Adjusting the SPR response based on molecular weight of the small molecule may be a source of error, although this error is generally not more than 2-fold.¹³ It is also formally possible for compounds, especially small ones such as needles or fragments,¹⁴ to bind two or more independent binding sites on a target protein, resulting in a stoichiometry above 1:1. Thus, we use this classification to indicate some nonoptimal behavior, the severity of which needs to be addressed by retesting at higher concentrations if solubility allows or by characterization in a different assay such as substrate competition or X-ray crystallography.

Concentration-Dependent Aggregation. A third major type of undesirable compound binding behavior is concentration-dependent aggregation (Figure 3D). This behavior is distinguished from those described above because at lower concentrations these compounds appear well behaved and are either nonbinding or bind reversibly and at subsaturating levels. However, increasing the concentration as little as 2- or 3-fold

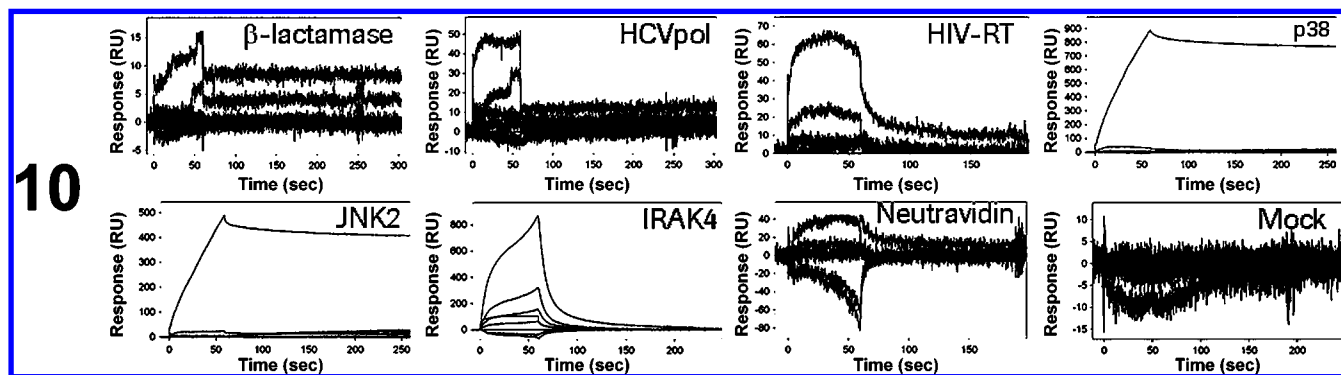


Figure 4. Protein identity can influence the type of observed binding. Compound **10** shows almost no binding to β -lactamase or neutravidin but does show small amounts of binding to HCVpol and HIV-RT (nonstoichiometric). Binding is superstoichiometric to all the kinases (p38, JNK2, and IRAK4) but is only reversible in the case of IRAK4.

results in the observed signal rising beyond that expected for a 1:1 interaction (Figure 3D, compound **8** and p38). Often the amount of increase is far beyond that expected for the population of one or two additional binding sites on the protein (Figure 3C, compound **9**, IRAK4). Above the concentration threshold, these compounds aggregate and bind with the properties described above (e.g., enveloping the protein) or compound monomers begin to nonspecifically associate in many places on the protein surface, creating large jumps in response that do not represent the desirable site-specific binding properties of druglike leads. Concentration-dependent aggregators can adopt the properties described above for non- or superstoichiometric binders and/or change from reversible to irreversible binding. Concentration-dependent aggregation was fairly common in our experiments but could be difficult to spot because often only the top one or two concentrations were affected. It was useful to compare these sensorgrams to a simulation of the expected behavior (Figure 3D, JNK and **10**, compare with simulation in inset) or attempt to fit the data to a 1:1 model and examine the fit parameters to discern if the compound behavior changed at higher concentrations.

Reversibility. SPR experiments consist of a real-time observation of the association and dissociation phases of a molecular interaction, allowing us to assess the interaction's reversibility. Many of the interactions showed a very slow release from the surface, indicating that the interaction was functionally irreversible (e.g., Figure 3A, JNK2). The qualitative shape of the dissociation phase for these compounds is suggestive of nanomolar to picomolar affinity, which is several orders of magnitude more potent than expected for a ligand prior to optimization. These observations are consistent with interpretations of biochemical inhibition data suggesting that some aggregates interact very tightly with proteins. Covalent binding is not a likely cause of this apparently irreversible binding because the binding is often detergent sensitive, and the compounds chosen are not likely to be chemically reactive, especially given the high concentrations of dithiothreitol in the experiment. Some compound/protein combinations dissociate to baseline quickly (Figure 3C, IRAK4), suggesting that they exist in a dynamic equilibrium. These aggregates either have a lower affinity for protein or are not stable aggregates and dissociate or dissolve quickly as the concentration falls during the dissociation phase. For example, a detergent-like model would predict that the micelle breaks into monomers as the concentration during washout falls below the critical micelle concentration (CMC).

Detergent Sensitivity. Previous reports establish that promiscuous binders lose their inhibitory properties in the presence

of a variety of detergents.^{4,5,8} Compounds that exhibit this effect are labeled as detergent sensitive. To test this effect in our assay, all experiments were performed in buffers with and without 0.005% Tween-20 (70% of CMC)⁵ (GE Healthcare). A number of the compounds were sensitive to detergent and changed their behavior in the presence of the Tween-20. We frequently observed that undesirable binding in detergent-free buffer, such as an irreversible superstoichiometric binder, would exhibit reversible and subsaturating binding in the presence of detergent (Figure 3E, compound **10** and p38). Rarely did badly behaved compounds become well-behaved, with most still exhibiting nonstoichiometric binding at lower binding levels than in detergent-free conditions. Others simply showed no significant binding when detergent was added (Supporting Information Figure 1, compound **5** and β -lactamase). Detergent-induced reduction in binding is consistent with detergents preventing aggregate/protein interactions or compound self-aggregation and thus eliminate nonspecific inhibition of enzyme activity. We note that our data contain cases where detergent has no effect on the binding profiles (Figure 3E, compound **9** and HCVpol), indicating the potential for false-negatives in assays that identify promiscuity based solely on detergent sensitivity. In a few cases detergent actually made the compound behave worse (Supporting Information Figure 1, compound **9** and neutravidin), although these incidents were rare. Detergent sensitive interactions are marked in bold in Supporting Information Table I.

Effect of Different Proteins on Compound Behavior. A surprising observation in our data is that some compounds' behavior varied depending on the protein surface. While most compounds behaved similarly across all tested surfaces (see Supporting Information Figure 1), some compounds exhibited one type of badly behaved binding against one protein and a different class of behavior against another protein (in Figure 4 compare HIV-RT and p38). Surprising, though more rare, is the case where a compound is badly behaved against one protein but is nonbinding or well behaved against a different protein (in Figure 4 compare JNK2 and neutravidin). The mechanism for this differential aggregation on proteins cannot be determined solely from these data, although it would suggest that different proteins are able to promote compound nucleation to different degrees, even within the same class of proteins (in Figure 4 compare the kinases p38 and JNK2 to IRAK4). Similarly, the physiochemical parameters of some aggregates may be better tuned to recognizing certain protein shapes and electrostatic profiles inherent to some proteins but not others. Therefore, observation of undesirable binding properties of a compound against one protein may not be sufficient to broadly classify it

as a promiscuous inhibitor, as its behavior may be significantly different with other proteins.

Exposure to Promiscuous Compounds Does Not Damage Proteins. The protein surface in an SPR experiment is used repeatedly through the course of an experiment. If a compound or solution is injected that damages the protein or surface, then the chip cannot be used further. To establish the utility of the SPR experiment as a screening technique to identify promiscuous binders, it was important to demonstrate that the protein surface is stable despite repeated exposure to badly behaved compounds. We interrogated the protein surface stability by comparing control compounds before and after exposure to 96 injections at various concentrations of the 13 promiscuous binders (compound **12** was run in duplicate). The R_{\max} values from fits to the controls, normalized to the response level of the first control series in the experiment, are presented in Supporting Information Table 1. HCVpol (Figure 2), JNK, and RT showed up to a 36% loss of binding capacity, while the other surfaces (IRAK4, p38, and JNK2) exhibited less than a 10% drop in R_{\max} . Inclusion of detergent moderates the decay (HCVpol) or eliminates it entirely (HIV-RT and JNK2) and may suggest a preference by those proteins for the presence of detergent, rather than damage inflicted by exposure to the compounds, or overall better compound behavior in the presence of detergent. In general, the loss of binding capacity by the surfaces was small, despite the fact that some of the test compounds bound irreversibly to a level 140 times the expected R_{\max} . The large signals were due to the size of the aggregate and not due to the saturation of the surface. Since the SPR signal is proportionate to mass,¹¹ we suppose that during the relatively short time of the experiment (1 min exposure compared with 20 min preincubation times in plate-based assays) and low concentration of aggregate relative to expected concentration of monomer, a large aggregate or micelle would only bind to few proteins on the surface and yet give a large change in signal due to the size of the aggregate, leaving the rest of the proteins untouched. Thus, any damaged or occluded protein molecules represent only a small fraction of total protein on the chip surface. These data indicate that, in general, protein surfaces are sufficiently stable to investigate the behavior of numerous poorly behaved compounds, especially in the presence of detergent.

Discussion

We report on the development of an SPR-based assay for detecting and characterizing promiscuously binding compounds that may act as false positives in biochemical assays. Traditionally, SPR assays have been primarily used in drug discovery to determine the affinity of small-molecule/protein interactions.¹⁵ However, SPR data also provide information about stoichiometry, reversibility, and changes in compound behavior over a range of concentrations. We leverage this information by applying the SPR assay to hits from biochemical screening assays and rapidly determine if inhibition results by a specific or nonspecific interaction with the protein. This aids in selecting and prioritizing hits for characterization in other assays and/or chemical elaboration by medicinal chemists.

From the data in this study of 13 known promiscuous binders interacting with 8 surfaces, we have identified a variety of compound behaviors that can be subdivided into four main groups. On the basis of this, we have developed a classification system based on observations of binding stoichiometry, concentration-dependent binding, and reversibility of the interaction. This scheme can be used to make decisions about what

compounds to advance into subsequent stages of lead generation. We assign the code "ss" (superstoichiometric) to compounds that bind at an observed compound to protein stoichiometry of greater than 5:1 and eliminate them from hit lists. Compounds with a stoichiometry greater than 1:1 but less than 5:1 are labeled nonstoichiometric binders (ns) and deprioritized, although they can be followed up in other assays if there is sufficient interest in other properties of the compound. Concentration-dependent aggregators (cd) are eliminated from further consideration and are not good candidates for structure-based design because they will likely aggregate and nonspecifically associate with protein at the high compound concentrations required for X-ray crystallography. We also note that occasionally a hit does not interact with the protein of interest but perhaps inhibits the enzymatic assay by interacting with another component of the assay (e.g., binding the nucleic acid substrate in a polymerase assay). We classify these compounds as nonbinders (nb) and eliminate them for further follow-up studies because they do not interact with the target protein. For all experiments we note whether the interaction is reversible based on observations of the dissociation phase of the SPR experiment. Thus, an irreversible superstoichiometric compound would be labeled as "ss/i" whereas a reversible compound that exhibits concentration-dependent aggregations is identified as "cd/r". Often multiple behaviors occur simultaneously (e.g., a compound behaves well at low concentration but aggregates at higher concentrations, and the aggregate is superstoichiometric and irreversible). In these cases only the primary characteristic of the compound that contributes to its erroneous biochemical potency is reported. In the example it would be concentration dependence and thus reported as "cd/i." More detail on class assignments is provided in Supporting Information. This classification scheme has been applied to all of the data in Supporting Information Figure 1 and is shown in Supporting Information Table I. It is important to note that promiscuously binding compounds are a fraction of the total hits from an HTS screen. We have observed badly behaved compounds comprising between zero and 21% of hits in six HTS campaigns.

The mechanistic information about promiscuous compound binding obtained from the time resolution of the SPR experiment supports previous studies. The apparent kinetics of some of the compounds indicate they are very slow to achieve equilibrium, consistent with the observation of time-dependent inhibition by such compounds.³ Additionally, many compounds have an apparently slow off-rate, resulting in the designation of irreversible binding. While the binding is not truly irreversible, as is only the case for covalent interactions, the complexes are slow to dissociate, indicating very tight binding. While these data cannot be properly fit to a 1:1 binding model to determine kinetics or binding constants, the observations of the kinetics are qualitatively indicative of nanomolar to picomolar binding constants. This supports the interpretation of large Hill slopes and dependence of IC_{50} values on protein concentrations in biochemical inhibition assays that aggregates may have a very high affinity for proteins.⁶

The flow-cell based SPR assay showed that a number of promiscuous binders rapidly dissociated to baseline during the dissociation phase of the experiment, whereas previous plate-based studies required detergent to separate the complex.^{4,5} Carrier proteins, such as BSA, can prevent aggregate association with the target protein but cannot reverse it.⁹ These results, combined with our observation that the identity of the protein can change the kind of interaction behavior with the aggregate, suggest that each compound's aggregated form may have its

own unique set of physicochemical properties making it possible for it to interact better with some proteins than with others. This context-dependent behavior may limit the degree to which computational filters, designed to identify promiscuous binders based only on chemical features, can be developed.^{16,17}

We use the SPR assay to characterize the binding behavior of hits identified in primary biochemical screening campaigns. While data collection across a wide range of compound concentrations is helpful for compound classification, we find that single concentration screening at the highest concentration used in the primary biochemical assay yields sufficient information for unambiguous classification of compound behavior. Thus, the SPR assay as we have implemented it has the capacity to screen ~300 compounds per day. Most proteins are stable enough to survive several days of screening without the need to prepare new surfaces. Compounds that pass the SPR prescreen by binding to the target protein reversibly and stoichiometrically are then retested at multiple concentrations to determine the K_D , providing additional information for confirming and prioritizing compounds for follow-up studies such as X-ray crystallography and chemical elaboration by medicinal chemistry.

The time resolution of the SPR experiment has given some insight into the various binding mechanisms that promiscuously binding molecules employ to nonspecifically inhibit enzyme function. Important findings from these studies include observations that some of the interactions are spontaneously reversible, either due to weak interactions with the protein or to low stability of aggregate, and that a compound's behavior can change depending on the protein in the system. This last observation indicates that it could be difficult to systematically identify, flag, and remove promiscuous compounds from screening libraries. This is especially true of frequent-hitter analysis against multiple screens where a given compound may behave badly in some assays but be well behaved or nonbinding in other assays. Therefore, optical biosensors can be an important secondary assay to high-throughput screening for the elimination of promiscuous binders as false positives and also for characterization of the mechanism of valid hit compound/protein interactions as part of the lead generation process.

Experimental Protocol

Protein Production. AmpC β -lactamase was a generous gift from Dr. Brian Shoichet, UCSF. Neutravidin was purchased from Pierce and dissolved in 50 mM HEPES, 150 mM NaCl, and 0.05% sodium azide. All other proteins were produced and as previously described.^{18–22}

Compound Selection. We chose compounds for this study based on previous literature describing promiscuous binders (compounds **2–5**, **7**, **10**, **12**, **13**)^{3,4,8,17} as well as from identification of frequent hitters from a number of in-house HTS screens (**1**, **6**, **8**, **9**, **11**). Control compounds were chosen from in-house projects or the literature²³ because they exhibit measurable kinetics and dissociate to baseline within a few minutes.

Binding Experiments. SPR experiments were performed with a Biacore S51 or a Biacore 2000 biosensor instrument (GE Healthcare) (see Supporting Information). Interactions between a protein immobilized on a biosensor chip and a compound flowed over the surface are monitored in real time as a change in surface plasmon resonance as measured in resonance units (RU).^{11,12} All proteins were biotinylated and captured to a neutravidin surface or directly amine-coupled (see Supplemental Methods in Supporting Information). A “mock surface” was also prepared by subjecting a surface to the amine-coupling procedure but with no protein coupled.

Prior to use, 10 mM stock solutions of compounds were diluted in DMSO to 1 mM and then diluted 20-fold into 50 mM HEPES

8.0, 150 mM NaCl, 10 mM MgCl₂, and 5 mM dithiothreitol to achieve a compound concentration of 50 μ M in 5% DMSO. Control compounds were diluted in 100% DMSO to a concentration of 20 times the K_D before dilution into running buffer. Control experiments (Figure 2 and Supporting Information Figure 1) verified that all tested proteins were active and stable under the chosen experimental conditions. To test the effects of detergent on compound behavior, some experiments included 0.005% Tween-20 (GE Healthcare). All experiments were run at 20 °C. No surface regeneration strategies were employed because of the general difficulty of identifying small-molecule/protein regeneration conditions and of assessing the effect of irreversible compound binding on the long-term binding stability of the surfaces. Binding properties of control compounds to their respective proteins in this buffer were similar to those of previous in-house studies (data not shown) or to literature results.²³

Data Processing. Raw sensorgram data were reduced, solvent-corrected, and double-referenced using the Scrubber II software package (BioLogic Software, Campbell, Australia; <http://www.biologic.com.au>). Control compounds were fit to a 1:1 binding model, with or without a mass-transport term as appropriate, using Scrubber II. For easy comparison between data sets, all experiments were normalized to an R_{\max} of 100 RU using a normalization formula based on experimental parameters (see Supplemental Methods in Supporting Information).

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Supporting Information Available: Additional details on experimental methods and the compound classification procedure and a complete data set and classification assignment for each interaction. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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