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biology at work

A Guide to Navigating Hit Prioritization After Screening Using Biochemical Assays

So you have performed your screen. What's next? This guide is focused on how biochemical assays are used for characterizing and prioritizing compounds following a primary screen with an enzyme target, whether using high throughput screening (HTS) or virtual screening (VS). A typical screening funnel is shown in Fig. 1, with the many applications of the biochemical activity assay highlighted. Here we will discuss strategies for hit-to-lead selection including: assay considerations, running a dose-response, hit confirmation, triaging, hit expansion, mechanism of action, and residence time studies.

Note: The workflow following a primary screen with a cellular HTS assay is likely to differ considerably. Cell-based functional assays are also very important to lead development post biochemical HTS, but are not highlighted by this guide.

Know Thy Target

There are myriad things to consider during a screening and/or hit-to-lead campaign, but a critical factor for success is having a reliable source of highly purified, catalytically active target protein. Regardless of whether it is produced internally or outsourced, it is imperative to ensure that the methods for the production of functional target protein are well established. This means carefully validating a batch produced at a scale sufficient to support your screening campaign, or at least enough for the biochemical assays used for HTS, hit prioritization, and hit-to-lead. If the same protein construct is used for biophysical analyses (SPR and TSA) and crystallography, these quantities need to be taken into account as well. Relying on validation of a smaller batch is a very risky proposition, as scaling up protein expression and purification is often not a linear process. In our experience, problems with the quantity or quality of target protein are the most frequent cause of delays and interruptions in lead discovery programs.

Initial testing should include assessment of purity using SDS-PAGE and measurement of catalytic activity with the primary assay. Functional stability should be tested by assaying activity following incubation at various temperatures (room temp, 4°C, on ice) and following several cycles of freeze-thawing. Though more stringent tests are available, identity should be confirmed by mass determination with the whole protein and by Western blot. It is important not to rely on vendors for this critical information. Additional testing might include measurement of substrate K_m values and IC_{50} values for known inhibitors, when possible.

Note: The K_m and specific activity of the protein will, to a large extent determine the sensitivity requirements of the assay and the quantity of protein required for screening and inhibitor profiling, and is, therefore, a calculation well worth making before initiating a discovery program.

After Screening What's Next

This guide is focused on steps following a screen, whether it be a virtual or a biochemical HTS. The following graphic (Figure 1.) provides an example of how a screening funnel can take shape. This guide will discuss each of these steps in detail providing examples of how to sort through your hits to find a bona fide inhibitor.

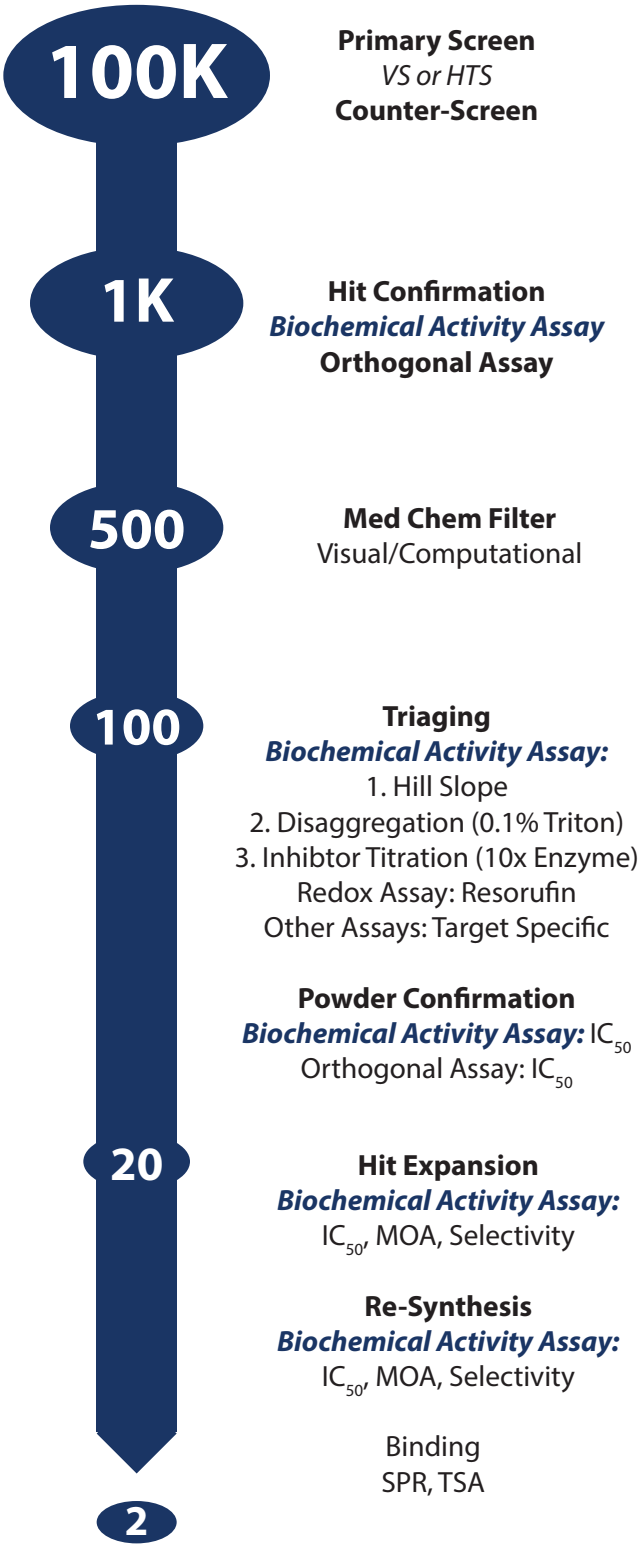


Figure 1. *Representative screening funnel highlighting frequent use of a biochemical activity assay which is often also used for the primary screen.*

Assay Considerations

Biochemical activity assay - The workhorse of any small molecule discovery effort is a robust biochemical assay for measuring enzyme activity. The requirements for an activity assay for hit-to-lead, listed below, are very similar to those for the primary screening assay, which is often used for hit-to-lead as well. However, a key difference is that the assay must be used in a way that provides a quantitative measure of product formation. Most HTS assays are capable of this, but it usually requires conversion of the raw signal to product formation using a standard curve, which is not generally done for a screen.

- **Sensitivity:** Should be sufficient for detecting initial velocity; i.e., detection of product at 10% or less of the initial substrate concentration. This is an absolute requirement for accurate determination of key parameters like IC_{50}, which are based on kinetic assumptions including minimal depletion of substrate. Note that screening is typically performed with substrate concentrations at or near the K_m, so enzymes with lower K_m values require more sensitive assays. For example, measuring initial velocity for an enzyme with a K_m of 100 nM (like some methyltransferases) will require an assay capable of detecting product at concentrations in the 2-10 nM range with good signal:noise; this is not trivial.

- **Homogenous**: Ideally, the assay should be homogenous; i.e., mix-and-read, to facilitate workflow and/or automation. Use of heterogenous methods such as LC-MS can be highly quantitative, however they are more cumbersome and time-consuming, and the constraints on resources can end up limiting the scope of mechanistic studies.

- **Detection Mode:** Commonly used HTS readouts compatible with a multiwell plate reader; e.g., fluorescence intensity, fluorescence polarization, TR-FRET, or luminescence are convenient. Absorbance based assays can certainly be used, but there will be more interference from colored compounds.

- **Endpoint vs. Continuous Detection:** Most analyses, including dose-response curves, are generally performed using endpoint detection. However, as described below, some types of mechanistic studies require running assays in continuous mode. If necessary, different assays can be used for dose response and mechanistic studies, though IC_{50} values obtained with the two methods should be compared carefully.

Aside from these requirements, the simpler the assay, the better; i.e., try to minimize number of detection reagents and/or steps required to convert the enzyme product into a signal. Each additional reagent and/or signal development step increases the potential for interference and/or pipetting errors. For example, kinase assays that rely on direct interaction of a phosphopeptide with an antibody or other affinity reagent (Fig. 2. A,B) are preferable over coupled-enzyme assays that rely on enzymatic conversion of the phosphorylated peptide for signal generation (Fig. 2. C).

In the latter case, test compounds may interfere with the coupling enzyme, whereas they are very unlikely to affect antibody binding. Similarly, for kinase assays that rely on ADP detection, direct detection of ADP (Fig. 2. D) is preferable to assays that use a complex coupled-enzyme cascade, requiring multiple development reactions, to convert the ADP into a signal (Fig. 2. E).

Another important consideration is whether the assay can be used with native substrates; this is an especially important consideration for enzymes that catalyze post-translational modifications (PTM) of proteins; e.g., kinases, methyltransferases, etc. Though the use of peptide substrates is cheaper and usually a reasonable choice, especially for screening, there is a risk that the kinetic parameters may be different than with the native substrate, and/or that some types of inhibitors may be missed. And there are cases, especially for methyltransferases, where an intact protein substrate is required for catalytic activity (1).

The use of a generic assay method; e.g., detection of ADP or SAH for kinases and methyltransferases, respectively, allows the use of either peptide or native protein substrates, and is therefore a good choice for PTM enzymes, especially if both types of substrates will be used during lead discovery.

Generic assays can also simplify selectivity profiling; however, it is not critical that the same assay be used for HTS, hit-to-lead, and selectivity profiling.

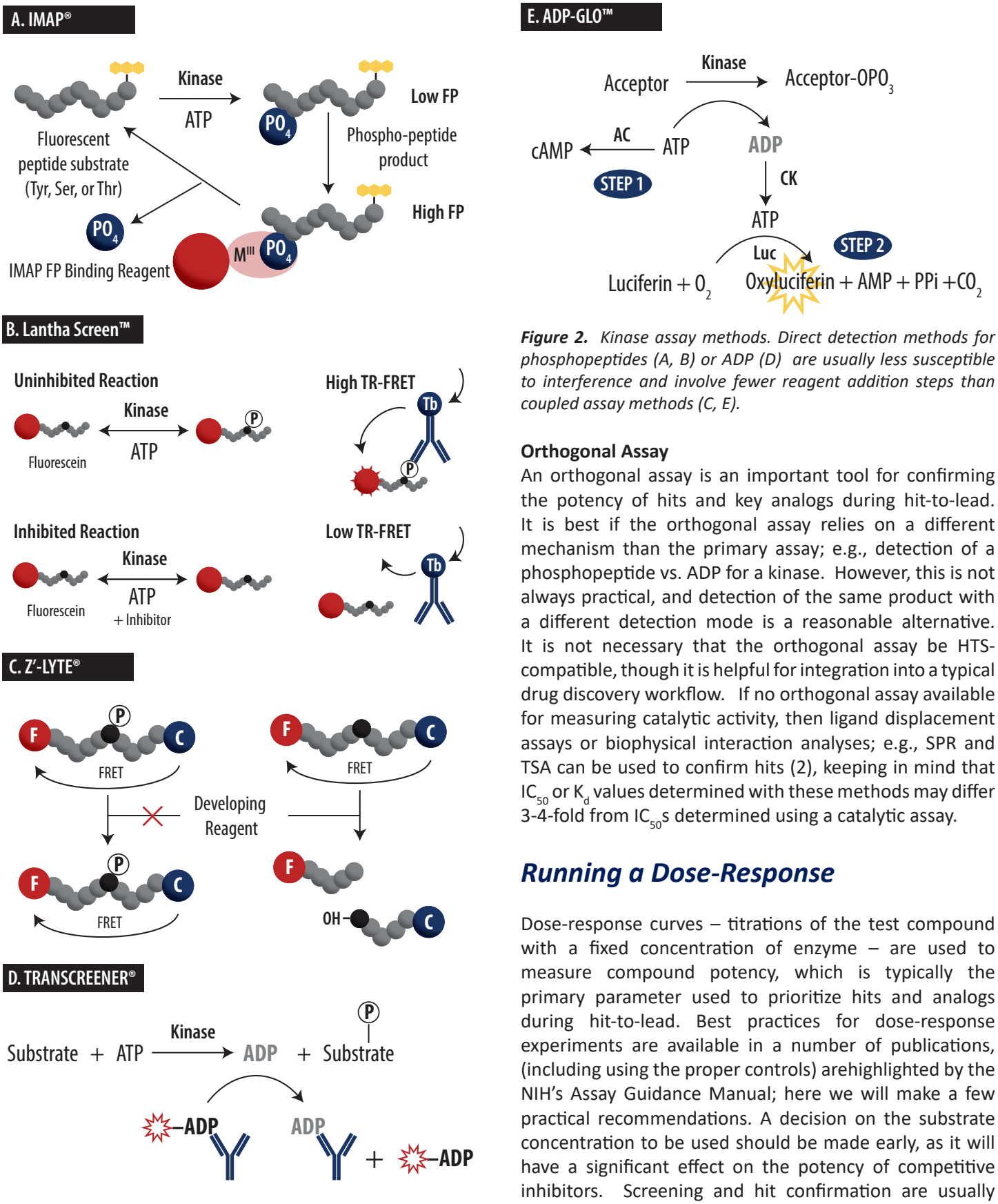


Figure 2. *Kinase assay methods. Direct detection methods for phosphopeptides (A, B) or ADP (D) are usually less susceptible to interference and involve fewer reagent addition steps than coupled assay methods (C, E).*

Orthogonal Assay

An orthogonal assay is an important tool for confirming the potency of hits and key analogs during hit-to-lead. It is best if the orthogonal assay relies on a different mechanism than the primary assay; e.g., detection of a phosphopeptide vs. ADP for a kinase. However, this is not always practical, and detection of the same product with a different detection mode is a reasonable alternative. It is not necessary that the orthogonal assay be HTS-compatible, though it is helpful for integration into a typical drug discovery workflow. If no orthogonal assay available for measuring catalytic activity, then ligand displacement assays or biophysical interaction analyses; e.g., SPR and TSA can be used to confirm hits (2), keeping in mind that IC_{50} or K_d values determined with these methods may differ 3-4-fold from IC_{50}s determined using a catalytic assay.

Running a Dose-Response

Dose-response curves – titrations of the test compound with a fixed concentration of enzyme – are used to measure compound potency, which is typically the primary parameter used to prioritize hits and analogs during hit-to-lead. Best practices for dose-response experiments are available in a number of publications, (including using the proper controls) arehighlighted by the NIH's Assay Guidance Manual; here we will make a few practical recommendations. A decision on the substrate concentration to be used should be made early, as it will have a significant effect on the potency of competitive inhibitors. Screening and hit confirmation are usually performed using the K_m concentration of substrate. After

this point, physiological concentrations of substrate should be used, if not for all dose-response experiments, then at least for compounds that look promising. This is necessary if the IC_{50} values are to reflect the potential potency of compounds in the cell. For example, a kinase inhibitor with 50 nM potency at 10 µM ATP may have little or no cellular activity because intracellular ATP concentrations are millimolar (3). A good practice is to use concentrations of test compound, at half-log (3-fold) intervals. Ideally, curves should plateau in both low and high concentration ranges for accurate IC_{50} measurements. Lack of complete plateau in the high concentration range can be dealt with to some degree with graphing software, by extrapolating to zero activity. This needs to be used with caution, as some compounds do not inhibit completely, and it is often necessary to repeat the experiment over a different concentration range for accurate estimation of IC_{50}s. It may not be possible to generate complete curves using compounds with very low potency and/or limited solubility, and typically an arbitrary cutoff point is established. If a curve plateaus in the high concentration range, but inhibition is less than 75%, the compound may have an undesirable mechanism and should be viewed suspiciously.

Hit Confirmation

Screening hits should be confirmed first using the primary screening assay, with replicates at two or three concentrations or full dose-response curves, and then using an orthogonal assay, if available, also in dose-response mode. After triaging, the remaining hits should be purchased as powders and reconfirmed with both assays using dose-response measurements.

Triaging

Structural Alerts

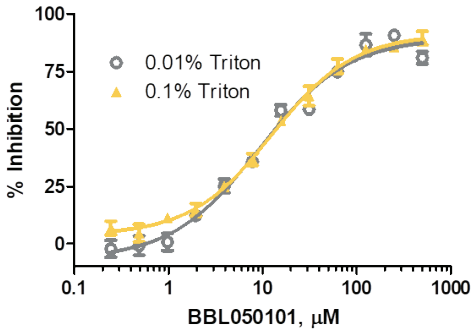
An experienced medicinal chemist can easily examine a few hundred hits to eliminate compounds with reactive groups or obvious metabolic liabilities, many of which will be clustered. Visual analysis can be employed at an intermediate time point in hit confirmation; this is especially helpful if the throughput of the orthogonal assay is limiting. There is no single best approach for every situation; it depends to some degree on the total number of hits and the throughput for the orthogonal assay.

Non-stoichiometric Inhibitors

Identifying and triaging non-stoichiometric inhibitors (NSIs) is a critical early step in hit-to-lead, as these artifactual compounds can constitute more than half of the hits from commercial compound libraries (4, 5). It is not safe to rely on computational filters for removal of NSIs; they need to be identified experimentally. There is a substantial literature on NSIs, and some common mechanisms such as compound aggregation have been described (4, 5), but in the end, what is clear is that molecules that bind non-stoichiometrically are unlikely to yield a drug. There are three indicators that can be used to flag NSIs:

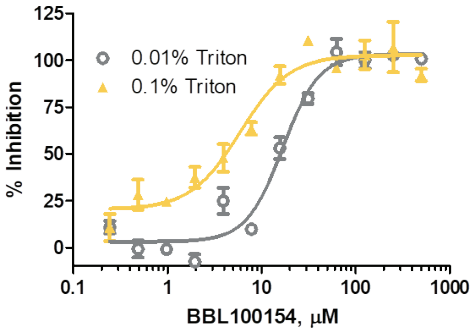
- **Disaggregation:** A leftward shift in the dose-response curve when the assay is run in the presence of 0.1% Triton X-100 indicates that the compound is acting as an aggregate (6); the detergent dissociates the aggregates, thereby increasing the effective concentration of the inhibitor.

A.



	0.01% Triton	0.1% Triton
HillSlope	0.9247	1.003
IC50	9.261	12.61

B.

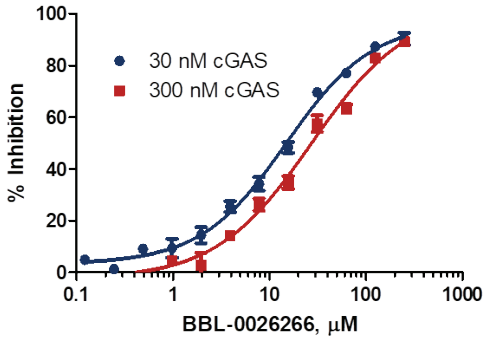


	0.01% Triton	0.1% Triton
HillSlope	2.254	1.654
IC50	16.75	5.903

Figure 3. *Disaggregation assay for identification of NSIs. Addition of a sufficient concentration of non-ionic detergent (0.1% Triton X-100) disperses aggregated inhibitors, resulting in a leftward shift in the dose response curve (B); 'normal' inhibitor (A).*

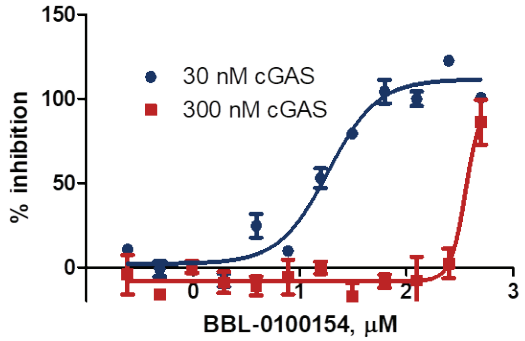
- **Inhibitor titration:** A rightward shift in the dose-response curve when the concentration of enzyme is increased 10-fold indicates that the compound is acting non-stoichiometrically (4); the higher concentration of enzyme 'titrates' aggregated or covalent inhibitors, effectively lowering the free concentration and shifting the equilibrium. Keep in mind that reaction times may have to be adjusted accordingly to maintain initial velocity conditions.

C.



	30 nM	300 nM
HillSlope	0.9751	0.8742
IC50	14.98	27.23

D.



	30 nM	300 nM
HillSlope	1.880	6.137
IC50	18.54	359.5

Figure 4. *Inhibitor titration: A significant increase in IC_{50} (≥ 3x) when enzyme concentration is increased indicates an NSI (D); 'normal' inhibitor (C).*

- **Hill Slope:** If the Hill slope of the dose-response curve is greater than 1.6-1.7 one should be suspicious, especially if this occurs with more than one assay method. This alone is not reason to triage a compound, as allosteric inhibitors will show higher hill slopes (17).

Redox-Active Inhibitors

Oxidation of metalloenzymes or enzymes with catalytic cysteines is generally considered a relatively non-specific inhibitor mechanism; these compounds can be identified using a simple fluorescence-based assay comprised of readily available reagents (7).

DNA Intercalators

For enzyme targets that are involved in sensing, metabolism, or modification of nucleic acids, it is important to triage DNA intercalators, as they tend to be highly promiscuous. Intercalating compounds can be identified using a fluorescence polarization-based assay (8).

Triaging Confirmation from Powder and Re-Synthesis

After triaging, the remaining hits should be purchased as powders and reconfirmed with both assays using dose-response measurements. At some point prior to biophysical binding studies, the most potent compounds of each promising chemotype should be synthesized and retested. Obviously, this can involve significant time and expense, and it may be more efficient to first perform some initial SAR from commercially available compounds to help prioritize.

Hit Expansion (analog by catalog)

Demonstrating some SAR using commercially available analogs (in dose-response mode) is a relatively inexpensive way to provide additional validation for hits, and it also yields information on potential scaffold hops and tolerance for modifications that can help with compound prioritization. All of the chemical suppliers have similarity searching built into their websites and will supply 2-3 mg quantities of most molecules for less than $50. The minimal information required is an IC_{50} determined using the primary assay; interesting analogs can be confirmed using other parameters, including IC_{50} with the orthogonal assay, testing for NSI, and comparing mechanism with the hit compound.

Biophysical Binding Studies

Aside from a co-crystal structure, analyzing ligand binding to a target using biophysical methods is the

most convincing way to validate hits, and it is critical for characterizing analogs during the hit-to-lead process. Though other approaches such as microscale thermophoresis are emerging, surface plasmon resonance (SPR) and temperature shift analysis (TSA) are the most common and widely accepted methods (2). It's important to understand how each method works and the type of information that they provide.

In TSA, proteins are subject temperature gradient in the presence of a fluorescent probe that is quenched by water. As the proteins unfold, their exposed hydrophobic domains bind the dye resulting in increased fluorescence until they began to aggregate and exclude the dye with a corresponding decrease in fluorescence. The melting curves provide a T_m for the protein, which is typically shifted by 1-5 degrees by a small molecule ligand.

Most ligands increase the target protein stability, and the concentration dependence of shift provides a qualitative indication of affinity. A compound that causes a shift to a lower T_m is destabilizing the protein, and should be carefully tested for NSI and/or effects on other enzymes, as it may be acting with an undesirable and/or non-specific mechanism. However, it should not be discarded out-of-hand, as it could be binding specifically to a less stable conformation which can be a desirable mechanism.

TSA is most frequently used as a binary assay; either the ligand binds, or it does not. When used in this way, it is easier and cheaper to scale up than SPR. It can be used quantitatively to measure K_d, but this requires relatively complex analytic methods (9). Aside from confirming ligand binding, TSA is very useful for profiling the effect of various agents on protein stability to guide to crystallization efforts.

SPR is a spectroscopic method in which a protein is immobilized to a metal film and the interaction with ligand is detected via a change in the reflected light. SPR provides more information than TSA, including both the kinetics (k_{on}, k_{off}) and affinity (K_{eq}) of ligand binding. However, immobilization of the protein can limit its conformational mobility and also may introduce complexities for proteins with more than one sub-unit and/or dissociable cofactors.

Concordance of affinity measured by SPR (K_d) with potency of inhibition in a biochemical activity assay (IC_{50}) provides strong validation for a compound as a bona fide inhibitor, though it is important to remember that the two parameters are not equivalent. As a rule thumb, a similar rank order with individual K_d and IC_{50} values within 3-4 fold of each other is indicative that a series of compounds is binding and inhibiting the target specifically.

Careful consideration should be given to the source and form of protein used for biophysical binding studies. If at all possible, the construct used for measuring inhibitor potencies should also be used for biophysical studies, so that K_d and IC_{50} values can be compared meaningfully. However, whereas it may be desirable to use a full length and/or multi-subunit protein for SAR and mechanistic studies, this may not be possible for SPR, and in this case TSA may be advantageous. Also, immobilization for SPR is simplified by the use of one of the common affinity tags; e.g., a His-tag.

Selectivity Profiling

Selectivity versus a few closely related enzymes, either in the same family or with similar substrates and/or binding domains, is a good way to help prioritize hits and early analogs. Still, it obviously has to be considered in context with other properties when deciding which compounds to advance. Keep in mind that a hit should not be expected to exhibit much selectivity versus closely related enzymes; 2-3-fold is a good result and 10-fold is very promising.

The availability of a suitable assay can be a practical limitation, and in this regard, assays that are generic for an entire family are advantageous; e.g., ADP detection for kinases. More extensive selectivity profiling across entire families and/or other target classes is generally performed later in lead development, and almost always is outsourced.

Mechanism of Action

The biochemical activity assay is used to determine whether a compound is competitive or not. Competitive inhibitors bind in the active site and displace the substrate; compounds that are not competitive usually bind elsewhere. Obviously, this information is important for developing the hit-to-lead and SAR strategy. The simplest diagnostic for a competitive inhibitor is a decrease in potency as substrate is increased: greater than a 3-fold increase in IC_{50} when substrate is increased from K_m

to 10 x K_m is a good indicator of a competitive inhibitor (10). Uncompetitive and non-competitive inhibitors typically bind somewhere other than the active site and their potency is not affected by substrate concentration. Uncompetitive inhibitors bind specifically to the enzyme-substrate complex, whereas non-competitive inhibitors bind non-specifically to the enzyme regardless of whether substrate is bound. Discerning between the two requires more detailed kinetic analysis, as described (10, 11), and may not be a critical factor for the hit-to-lead strategy.

Residence Time

Analysis of drug-target residence times is increasingly being used for prioritizing compounds during hit-to-lead because longer engagement with the target can result in improved efficacy, increased therapeutic window and reduced side effects (12). Residence time can be estimated as the reciprocal of the dissociation rate (k_{off}) of an inhibitor from its target. SPR is commonly used to measure k_{off}, however, throughput can limit its use for prioritizing compounds early in discovery.

An alternative is to use a classic "jump dilution" catalytic assay method for determination of k_{off} values (13, 14). Use of this approach require an assay capable of continuous mode detection. We have described a detailed protocol for jump dilution assays for kinases elsewhere (14).

Briefly, recovery of enzymatic activity is monitored after a rapid dilution of enzyme-inhibitor complex into a reaction mix that contains all of the assay components. Then k_{off} values are determined by fitting enzyme progress curves to an integrated rate equation.

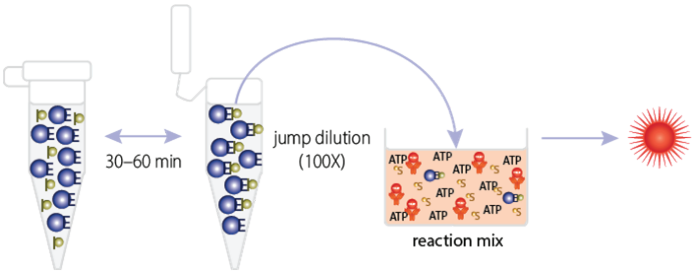
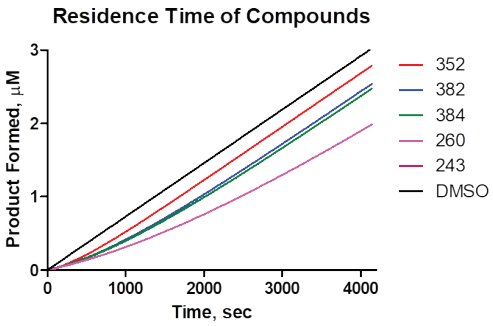


Figure 5. *Jump Dilution Method: Inhibitors are preincubated with enzyme at saturating concentration to allow formation of E-I complexes, then diluted 100-fold into reaction mix. Recovery of enzyme activity correlates with inhibitor dissociation.*



	352	382	384	260	243	DMSO
K	0.002126	0.001019	0.0008883	0.0003839	1.672E+13	1.65E+15
T1/2 (min)	7.84	16.36	18.76	43.41	<1	<1

Figure 6. *Monitoring recovery of activity for a series of inhibitors following jump-dilution. Activity recovers as the E-I complex dissociates, allowing calculation of off-rates.*

Conclusion

In any small molecule inhibitor program there can be an overwhelming amount of information. Sifting through "hits" whether it be via a virtual or biochemical screen requires the proper strategy and tools. One of the most useful of which is the biochemical assay. As a Swiss-Army knife of methods, there are many applications where a universal biochemical assay can help distinguish between real inhibitors and artifact compounds. Being able to measure enzyme products also affords the ability to screen inhibitors for their drug target residence time, adding an additional level of hit characterization that can be faster and cheaper than other methods. Finding the right compound from a screen takes much time and effort, with the help of some tools provided here we hope you can accelerate your drug discovery efforts.

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