CHAPTER SIX

FLiK: A Direct-Binding Assay for the Identification and Kinetic Characterization of Stabilizers of Inactive Kinase Conformations

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Abstract

Despite the hundreds of kinase inhibitors currently in discovery and preclinical phases, the number of FDA-approved kinase inhibitors remains very low by comparison, a discrepancy which reflects the challenges which accompanies kinase inhibitor development. Targeting protein kinases with ATP-competitive inhibitors has been the classical approach to inhibit kinase activity, but the highly conserved nature of the ATP-binding site often contributes to the poor inhibitor selectivity. To address this problem, we developed a high-throughput screening technology that can discriminate for inhibitors, which stabilize inactive kinase conformations by binding within allosteric

Methods in Enzymology, Volume 548
ISSN 0076-6879
http://dx.doi.org/10.1016/B978-0-12-397918-6.00006-9

pockets in the kinase domain. Here, we describe how to use the Fluorescence Labels in Kinases approach to measure the K_d of ligands as well as how to kinetically characterize the binding and dissociation of ligands to the kinase. We also describe how this technology can be used to rapidly screen small molecule libraries in high throughput.

1. INTRODUCTION

A major roadblock in protein kinase inhibitor research and development is the challenge of poor selectivity and the likelihood of unwanted off-target inhibition, which are largely a consequence of the highly conserved ATP-binding site shared by all protein kinases. Although drugs, which specifically target and inhibit the activity of a single kinase, are highly desirable, evidence is gathering, which suggests that polypharmacology may be beneficial in the fight against complex diseases such as cancer (Knight, Lin, & Shokat, 2010; Morphy, 2010). Polypharmacology strategies focus on the concept of "controlled unselectivity" to target a specific cluster of kinases within one or more specific pathways to achieve the desired therapeutic outcome. An analogous approach is combinatorial-personalized therapy using multiple kinase inhibitors to achieve the same goal.

Regardless of the strategy employed, it is becoming more evident that examining drug–target residence times will provide a more complete context for fully understanding kinase inhibitor selectivity *in vivo* (Copeland, 2011; Copeland, Pompliano, & Meek, 2006). Ideally, inhibitors should have high rates of association (k_{on}) and slow rates of dissociation (k_{off}) to maximize residence time with the target enzyme. In the case of kinases, an inhibitor, which appears to be relatively unselective *in vitro*, can be rendered more selective if it dissociates most slowly from the kinase of interest. Thus, medicinal chemistry efforts should not judge selectivity-based solely on inhibitor affinities (K_d) and potencies (IC_{50}) obtained from *in vitro* profiling of compounds against the entire kinome (Anastassiadis, Deacon, Devarajan, Ma, & Peterson, 2011; Copeland, 2011; Karaman et al., 2008). Although these types of studies undoubtedly contain valuable information and provide a solid groundwork for further inhibitor development, it is possible for a wide range of k_{on} and k_{off} values to result in the same overall affinity ($K_d = k_{off}/k_{on}$; Markgren et al., 2002). Thus, in addition to affinity, lead optimization strategies should consider the kinetic components contributing to affinity. By identifying aspects of ligand structure, which prolong k_{off} relative to k_{on}, medicinal chemistry efforts can be directed to facilitate the design of molecules with improved residence times with the desired kinase.

This approach would likely minimize the unwanted consequences of high-affinity off-target binding *in vivo*.

Emerging data suggest that the issue of kinase inhibitor selectivity can be addressed by moving away from classical ATP-competitive (Type I) inhibitors and targeting the DFG-out pocket with Type II and Type III inhibitors (Davis et al., 2011). The DFG-out pocket is adjacent to the ATP-binding site and is frequently referred to an allosteric pocket or kinase-switch pocket. In comparison to the ATP-binding site, the allosteric pocket is a more restrictive binding cavity and is only accessible upon a change in conformation. Therefore, this pocket tends to be less accessible to small molecules. However, the amino acids lining this pocket are much less conserved across the kinome, providing opportunities for additional H bonding and hydrophobic interactions between the kinase and ligands, which can bind within this pocket (Liu & Gray, 2006). Once bound, these factors tend to reduce the k_{off} of Type II and Type III inhibitors relative to k_{on} (Copeland, 2011). Thus, a logical methodology for improving kinase inhibitor selectivity by prolonging drug–target residence times should focus on the identification and kinetic optimization of ligands, which can bind preferentially to the DFG-out conformation.

The availability of the DFG-out pocket requires the kinase-activation loop to adopt a catalytically deficient conformation in which the ATP-binding site becomes partially occluded by the Phe side chain of the DFG motif (Backes, Zech, Felber, Klebl, & Müller, 2008a, 2008b; Rabiller et al., 2010). While the DFG-out conformation is more favorable in the unphosphorylated kinase, phosphorylation of the activation loop shifts conformational equilibrium to the more active DFG-in conformation, increases kinase activity, and often reduces the affinity of Type II and Type III inhibitors. Although the search for chemical scaffolds, which have affinity for the DFG-out pocket, is moving to the forefront of kinase inhibitor research, efforts have been constrained by the lack of high-throughput assay technologies, which can identify and discriminate for ligands which bind to and stabilize enzymatically inactive kinase conformations.

We have developed FLiK (Fluorescence Labels in Kinases) as a widely applicable assay system for both identifying and characterizing DFG-out-binding ligands. Kinases are site specifically labeled with an environmentally sensitive fluorophore, which reports on conformational changes induced by the binding of specific types of ligands (Fig. 6.1A; Simard, Getlik, et al., 2009; Simard, Kluter, et al., 2009). Changes in kinase conformation alter the charged microenvironment and solvation of the fluorophore, resulting

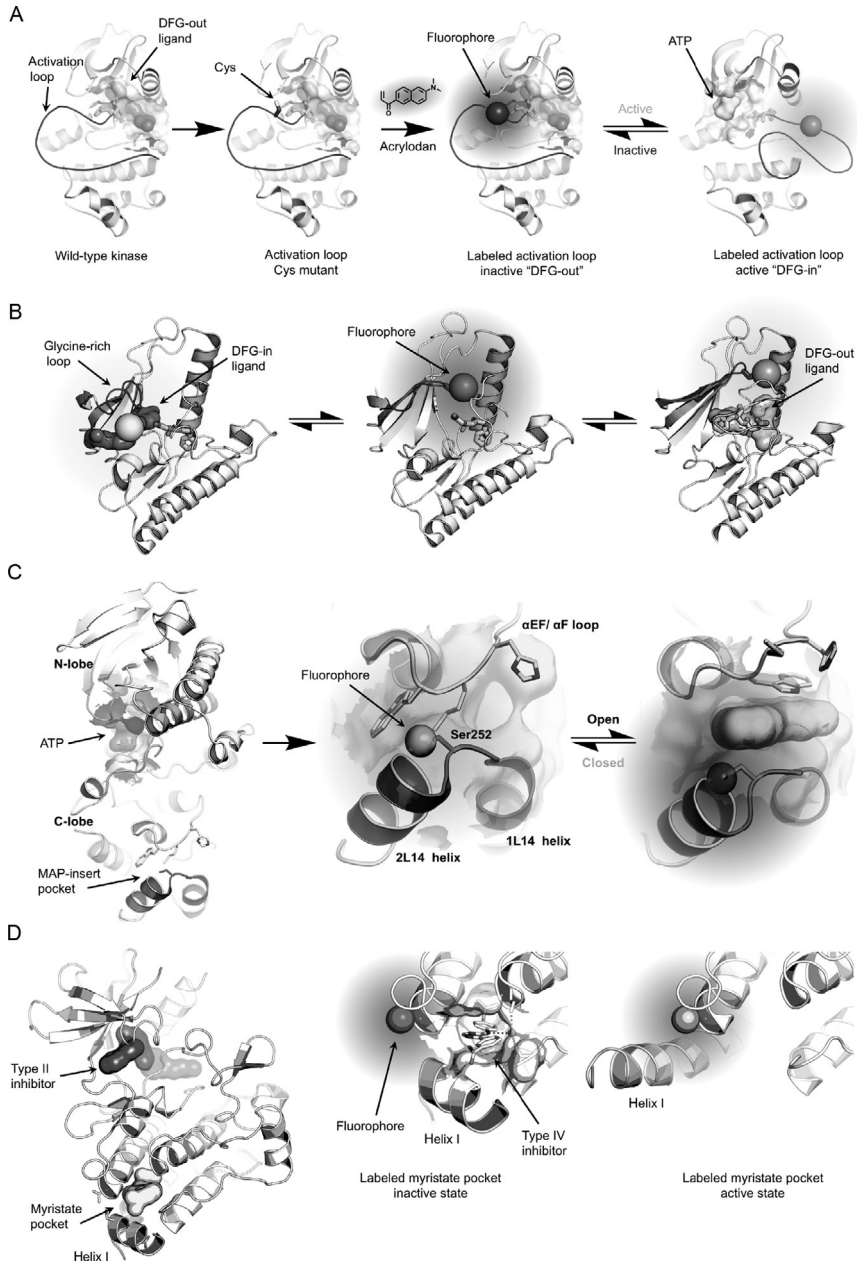

Figure 6.1 Overview of various FLiK assays for kinase-binding sites. (A) Labeling the activation loop. Kinases are regulated by an activation loop, which can adopt active (DFG-in) and inactive (DFG-out) conformations. As a general approach, a Cys residue is mutated into the desired labeling position and is used for the attachment of an

in distinct and quantifiable changes in its emission spectrum, which, in turn, provide a straightforward binding assay methodology for determining the K_d of the ligand. The assay also allows for follow-up characterization of identified compounds by permitting the determination of k_{on} and k_{off} to better understand the kinetic factors, which contribute to the measured K_d. A key advantage of this approach is that enzyme activity is not required. Using unphosphorylated kinase, the sensitivity for ligands, which bind preferentially to the DFG-out conformation, is enhanced when compared to measuring IC_{50} values using traditional activity-based assays, which rely on the use of phosphorylated active kinase.

The FLiK approach has been used to successfully monitor conformational changes in the activation loop of both Ser/Thr and Tyr kinases associated with the slow binding of DFG-out inhibitors (Grutter et al., 2012; Simard, Kluter, et al., 2009; Simard, Getlik, et al., 2009; Simard, Grutter, et al., 2009). To date, we have applied the FLiK approach to other kinase structural elements (Fig. 6.1B), including labeling of the P loop (glycine-rich loop) to identify more selective Type I ligands, which engage the flexible P loop in certain kinases (Simard et al., 2010). Additionally, we have recently reported a labeling strategies aimed at remote-binding sites outside of the ATP-binding cleft as a method for identifying more selective allosteric (Type IV) ligands, including assays for the MAP-insert pocket of p38α (Fig. 6.1C) as well as the myristate pocket of Abl kinase (Fig. 6.1D;

environmentally sensitive fluorophore (colored sphere). This FLiK assay enables specific detection of ligands, which bind to and stabilize the DFG-out conformation, such as Type II and Type III kinase inhibitors. (B) Labeling of the P loop. The selectivity of some ATP-competitive inhibitors can be improved by introducing chemical moieties, which are capable of engaging the P loop of certain kinases. (C) Labeling of the MAP insert. The MAP insert is a structural feature found in MAP kinases, cyclin-dependent kinases, and glycogen synthase kinase (Akella, Moon, & Goldsmith, 2008). This FLiK assay enables the specific detection of ligands, which occupy the MAP-insert pocket of a selected group of kinases. Although it is not yet clear if binding to this site effects catalytic activity of the kinase, it may alter signaling cascades by modulating the docking and/or scaffolding interactions of the MAP insert with other proteins. (D) Labeling the Abl myristate pocket. Stable binding to this site requires the bending of helix I of Abl. This FLiK assay enables the specific detection of allosteric Abl inhibitors. *Panel (A): Reproduced from Simard, Kluter, et al. (2009) with permission from Nature Publishing Group. Panel (B): This panel has been reprinted with permission from Simard et al. (2010). Copyright 2010 American Chemical Society. Panel (C): This panel has been reprinted with permission from Getlik et al. (2012). Copyright 2012 American Chemical Society. Panel (D): This panel has been reprinted with permission from Schneider et al. (2012). Copyright 2012 American Chemical Society.*

Getlik et al., 2012; Schneider et al., 2012). However, these strategies are beyond the scope of this chapter. To highlight the potential of FLiK as an high-throughput screening (HTS) method for rapidly identifying slow-binding DFG-out ligands, we will describe its application to the activation loop of p38α in more detail and provide a step-by-step protocol for researchers who are interested in evaluating their own kinase targets.

2. DESIGN AND PREPARATION OF KINASES FOR FLiK

The FLiK approach requires the removal of solvent-exposed cysteines and the insertion of a cysteine into a desired position in the kinase, which will serve as the attachment point for a thiol-reactive fluorophore. The kinase mutant is then expressed and purified, labeled, and characterized using standard biochemical and biophysical methods. However, it is necessary to have a strategy in place when designing the protein construct. Identifying optimal fluorophore-labeling positions is critical to enabling a high-throughput assay that reports specific ligand-induced conformational changes.

2.1. Selection of the labeling position on the activation loop

Fluorophores such as acrylodan are commonly employed for generating fluorescent protein conjugates which report on conformational changes (Copeland, 2011; de Lorimier et al., 2002; Richieri, Ogata, & Kleinfeld, 1999). Ideally, fluorophores should be highly sensitive to polarity and/or the charged microenvironment that is characteristic of nearby amino acid chains in the protein. It is also advantageous to choose fluorophores, which are thiol reactive. In contrast to amine-reactive probes, labeling by thiol-reactive fluorophores is typically complete and more specific due to the lower abundance of free thiols in proteins. However, to ensure the specificity of labeling for the FLiK assay, naturally occurring Cys residues, which are solvent exposed, should be mutated away, where possible, leaving the inserted Cys as the only solvent-exposed anchor point for the fluorophore.

For the FLiK assay, it is critical to insert a Cys residue into an amino acid position on the kinase that exhibits significant movement upon ligand binding and is somewhat solvent-exposed to enable the covalent attachment of the added fluorophore. To date, we have reported on several labeling strategies, which we have successfully used to develop FLiK assays for various kinases. These assays enable rapid and specific detection of inhibitors and ligands with unique binding modes (Getlik et al., 2012; Grutter et al.,

2012; Schneider et al., 2012; Simard, Grutter, et al., 2009; Simard et al., 2010; Simard, Getlik, et al., 2009; Simard, Kluter, et al., 2009). Although the specific labeling site may vary for each kinase or binding site, a general approach can be applied in the design of kinase constructs compatible with the FLiK approach. For any structural feature, which is known to exhibit flexibility or undergo conformational changes upon ligand binding, a kinase construct, which is compatible with the FLiK assay, can be designed as follows:

(1) Perform a BLAST search using the full amino acid sequence of the kinase of interest as a method of finding other kinases with the highest percentage of sequence identity.

(2) Using PyMol (DeLano, 2002), analyze protein crystal structures of the kinase of interest, if available. If no published structures are available, several online-modeling tools such as Swiss Modeller (Arnold, Bordoli, Kopp, & Schwede, 2006) or ESyPred3D (Lambert, Leonard, De Bolle, & Depiereux, 2002) can be used to generate 3D structural models based on available structural templates in the Protein Data Bank (http://www.pdb.org). The kinases identified in the BLAST search may serve as convenient starting points.

(3) Using online tools such as Clustal W, perform amino acid sequence alignments of the kinase of interest with a number of other highly homologous kinases. This method may help identify positions in the sequence, which are compatible with the FLiK approach. Sequence alignments may help identify highly conserved regions, common phosphorylation sites, or positions known to be involved in key structural interactions. Additionally, sequence alignments may reveal certain positions, which have a naturally occurring Cys. Such positions may tolerate mutations in which a Cys is introduced for specific labeling with the desired fluorophore.

2.2. Preparation of p38α MAP kinase construct for FLiK

The FLiK assay was initially developed as an assay, which discriminates for Type II/Type III ligands, which bind to and stabilize the inactive DFG-out conformation of the kinase-activation loop (Simard, Getlik, et al., 2009, Simard, Kluter, et al., 2009). To date, the FLiK assay has also been utilized to monitor several types of ligand-induced conformational changes and different binding sites in p38α as well as other kinases (Getlik et al., 2012; Grutter et al., 2012; Schneider et al., 2012; Simard, Getlik, et al., 2009;

Simard, Kluter, et al., 2009; Simard et al., 2010). The expression and purification of a p38α MAP kinase FLiK construct is described later as an example.

2.2.1 Expression of p38α MAP kinase

For compatibility with the FLiK assay, the gene sequence for full-length human p38α was mutated by site-directed mutagenesis to introduce the desired mutations for specific labeling of the activation loop (C119S/C162S/A172C) (Simard, Getlik, et al., 2009). For assay development with other kinases, we have also successfully utilized synthetic genes generated with the appropriate mutations already incorporated into the DNA sequence. The mutant p38α gene was then cloned into an expression vector, transformed into *Escherichia coli* and expressed as described previously (Simard, Getlik, et al., 2009). The expression is accomplished as follows:

1. Clone the desired gene into a pOPIN-F expression vector.
2. Transform into BL21(DE3) *E. coli*.
3. Grow bacterial culture at 37 °C in LB media until an OD_{600} of 0.6 is reached.
4. Cool to RT in 30 min using a chilled water bath if necessary and induce protein expression with 1 m*M* IPTG.
5. Incubate the cultures overnight (~20 h) at 18 °C while shaking at 160 rpm.

2.2.2 Purification of p38α MAP kinase

His-tagged p38α was purified from *E. coli* as described previously (Simard, Getlik, et al., 2009). During purification, PreScission Protease was used to cleave the N-terminal His tag from the construct. Tagged kinase constructs may perform differently in the FLiK assay when compared to their cleaved counterparts. Full details of the purification are as follows:

1. Pellet the bacteria by centrifugation and then resuspend in Buffer A (50 m*M* Tris, pH 8.0, 500 m*M* NaCl + 5% glycerol + 25 m*M* imidazole).
2. Load sample onto a 30-mL Ni column, wash with at least three column volumes (CV) of Buffer A, then elute with a 0–50% linear gradient of Buffer B (Buffer A + 500 m*M* imidazole) over two CV.
3. Cleave the N-terminal tag by pooling the protein fractions and incubating with PreScission Protease (50 μg/mL final concentration) in a 12–30 mL capacity 10K-MWCO dialysis cassette overnight at 4 °C in

Dialysis Buffer (50 m*M* Tris, pH 7.5, 5% glycerol, 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT).

4. Centrifuge the protein for 15 min at ~13,000 rpm to remove any precipitate that may have formed during the cleavage step.
5. Collect and dilute the supernatant fourfold in Buffer C (50 m*M* Tris, pH 7.4, 5% glycerol, 50 m*M* NaCl, 1 m*M* DTT).
6. Load the sample onto a 1 mL Sepharose Q FF column and wash with 10 CV of Buffer C. Elute with a 0–100% linear gradient of Buffer D (Buffer C + 600 m*M* NaCl) over 20 CV.
7. Pool and concentrate the protein down to a volume of 2–4 mL using a 10-MWCO centricon.
8. Load the sample onto a Sephadex HiLoad 26/60 Superdex 75 column equilibrated with Buffer E (20 m*M* Tris, pH 7.4, 5% glycerol, 200 m*M* NaCl, 1 m*M* DTT) and elute at a rate of 2 mL/min.
9. Concentrate the eluted fractions of pure protein to ~10 mg/mL, then aliquot and store at −80 °C.

3. LABELING OF p38α MAP KINASE WITH ACRYLODAN

A wide selection of thiol- or amine-reactive fluorescent probes is commercially available (http://www.invitrogen.com) and can be used for the labeling of FLiK kinases. Given the lower abundance of cysteine in proteins, the use of thiol-reactive fluorophores is more straightforward and significantly reduces the number of sites available for nonspecific labeling. When choosing a fluorophore for establishing a reliable kinase biosensor for the FLiK assay, special consideration should be given to molecules, which are highly sensitive to changes in the charged microenvironment. These fluorophores will be the most sensitive detectors of conformational changes (de Lorimier et al., 2002). For example, acrylodan responds with a change in overall fluorescence intensity as well as a >40 nm shift in the emission maximum, thus enabling the use of ratiometric fluorescence readouts. Fluorescence ratios are widely used in assay development since they correct for subtle variations in sample volume across several samples. Further details regarding the fluorescence properties of acrylodan in the FLiK assay are provided elsewhere (Simard, Getlik, et al., 2009). A general procedure for labeling FLiK kinase mutants is described as follows:

1. Measure the molar concentration of the protein stock using traditional methods. Calculate the volume of acrylodan stock (prepared in DMSO) needed to achieve a 1.5:1 (mol:mol) ratio of acrylodan:protein.

2. Dilute the acrylodan stock in buffer until the DMSO concentration is <1% (v/v). Add the concentrated protein to the prediluted acrylodan. For thiol-reactive fluorophores, such as acrylodan, the chosen buffer should have pH < 8 and should not contain reducing agents such as DTT (*Note*: TCEP is compatible with the labeling of cysteine thiols). Refer to the manufacturer's instructions for ideal labeling conditions for the selected fluorophore.
3. Incubate sample on ice or refrigerate (4–8 °C) overnight. The labeling procedure may be optimized for each protein/fluorophore combination by altering the temperature and length of incubation. For example, the progress of the acrylodan-labeling reaction can be followed over time by monitoring increasing emission intensity between 460 and 510 nm (acrylodan excitation wavelength = 386 nm) under various conditions.
4. Remove the remaining unreacted fluorophore through repeated buffer exchange cycles using a 10-MWCO centricon. Concentrate, wash, and dilute the protein at least three times when using this method. For large quantities of protein, unreacted acrylodan can also be removed by passing the protein over an appropriate size exclusion column.
5. Concentrate the final washed protein sample and store at −20 to −80°C.
6. Subsequent analysis of the extent and specificity of labeling should be done using mass spectroscopy methods as described elsewhere (Simard, Kluter, et al., 2009).

4. ASSAY CHARACTERIZATION AND VALIDATION

Before using the newly labeled kinase in a costly HTS campaign to assay large compound libraries, the following list of tests should be performed to characterize the fluorescence response induced by ligand binding. Ideally, a reference ligand should be chosen, which is known to induce the desired conformational change. In the case of p38α labeled at the activation loop, Type II or III inhibitors were available as positive controls. Type I inhibitors were also useful as negative controls. These ligands can be synthesized based on the available literature procedures, purchased from commercial sources, or may be included in proprietary compound libraries.

Several quantifiable variables (ΔR_{max} and ΔI_{std}) can be determined to assess the quality of a fluorescent kinase as a biosensor (de Lorimier et al., 2002). However, the Z' factor is the gold-standard statistical measure of assay quality and is used to predict if assays will be useful for HTS. The Z' factor is calculated using the equation:

$$Z' \text{ factor} = 1 - \frac{3 \times \left(\sigma_p + \sigma_n\right)}{\left|\mu_p - \mu_n\right|} \tag{6.1}$$

where both the mean (μ) and the standard deviation (σ) of both the positive (p) and the negative (n) controls (μ_p, σ_p and μ_n, σ_n, respectively) are taken into account. It is important to examine these variables for every FLiK construct, since shifting the labeling position on a protein can have mild or drastic effects on the ligand-induced fluorescence response. The following types of measurements are recommended for thorough assay characterization and validation.

4.1. Measure emission spectra for each kinase conformation

Using reference inhibitors, which are known to induce specific conformational changes, the first test should focus on the emission spectrum of the FLiK kinase and how it changes in response to ligands, which induce the desired conformational change (Fig. 6.2A). It is also necessary to check various other compounds to determine whether the assay is capable of specifically detecting the desired subset of compounds with a certain binding mode. This can be done as follows:

1. Prepare a cuvette containing a mini stir bar and a suspension of 50–100 n*M* FLiK kinase in Assay Buffer and place the cuvette into the sample holder of the fluorescence spectrometer. If possible, the instrument should have magnetic plate under the cuvette to enable rapid sample mixing.
2. Using the appropriate excitation wavelength for the chosen fluorophore, scan fluorescence emission over a wide range of wavelengths to capture a full spectrum.
3. *Positive control*: Add an excess (10–20 μ*M*) of a reference ligand (any compound which is known to stabilize or bind to the desired kinase conformation) and stir the sample briefly to ensure full dispersion of the compound and DMSO (vehicle). Measure the emission spectrum repeatedly over a period of time until no further changes are observed in the measured spectrum.
4. Overlay the spectra series using graphing software to visualize the changes in emission spectra that occur with ligand binding over time.
5. Identify the emission maxima of the FLiK kinase in the absence of ligand and compare that to the spectrum obtained in the presence of a saturating amount of ligand. For each kinase conformation, use the emission

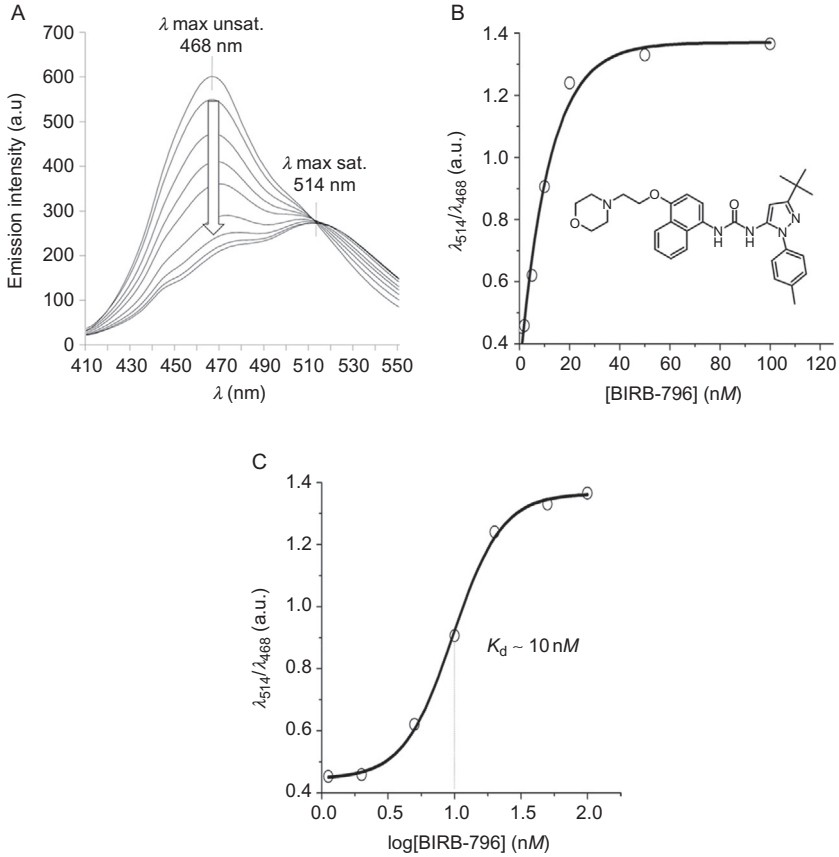

Figure 6.2 K_d determination for a DFG-out-binding ligand. This figure presents a typical set of endpoint measurement data obtained from the described K_d determination experiments. Shown data were obtained using acrylodan-labeled p38α and the Type II inhibitor BIRB-796. (A) The ratiometric fluorescence values are obtained from the emission spectra obtained in the presence of increasing inhibitor concentration. (B) These data can be plotted to observe the saturation of the kinase in a particular conformation or (C) these data can be used to directly obtain the K_d for the ligand. *Panels have been reprinted with permission from Simard, Getlik, et al. (2009). Copyright 2009 American Chemical Society.*

intensities at the two chosen wavelengths to determine the ratiometric output for the bound and unbound kinases. These values will give an indication of the lowest and the highest possible ratio values, or the "assay window." For ratiometric readouts, it is preferable to use fluorophores with at least two emission maxima or a single maximum, which shifts significantly (>10 nm) upon ligand binding. In the case

of acrylodan, one maximum does not change while the other is very sensitive to conformational changes in the protein.

6. Repeat the measurement several times to obtain multiple values for the unbound and bound states of the kinase. Use these values to calculate the Z' factor for the assay as shown in Eq. (6.1) earlier. The Z' factor should ideally be >0.5 for HTS.
7. *Buffer optimization*: If needed, adjust various buffer conditions to make the fluorophore more sensitive to conformation changes. Since environmentally sensitive fluorophores respond to local changes in charge and polarity, the magnitude and sensitivity of fluorescence change can sometimes be enhanced by adjusting ionic strength (salt concentration), pH, or by adding a detergent at an optimal concentration. Buffer optimization can be time consuming and should only be necessary if the initial response of the construct was found to be not robust enough for HTS.
8. *Negative control*: Repeat steps 1–5 above using any ligand, which is a known inhibitor of the kinase, but is known to bind in an alternate binding mode or binding site and does not induce the desired conformation change. Ideally, the FLiK construct should not respond to such ligands.

4.2. K_d determination

In the FLiK assay, K_d values are typically obtained from endpoint measurements in which ratiometric values of emission intensities at two different wavelengths are plotted as a function of inhibitor concentration (Fig. 6.2B and C). These experiments can be carried out using one of two possible methods. The most favorable method should be considered based on the knowledge of protein availability, required binding times for ligands, and stability of the kinase in buffer over time. In the first method, ligand is titrated into a single cuvette containing the labeled protein, which is rapidly stirred, and spectra are measured after each addition of ligand. The advantage to this approach is that it requires less protein to obtain a single K_d curve. However, the disadvantage is that this approach may be time consuming when a very slow-binding ligand is titrated with the kinase. For slow-binding ligands, a more preferable method for determining the K_d involves addition of a single dose of inhibitor added to a series of cuvettes at increasing concentrations. Following a long incubation time to ensure complete binding, each cuvette is measured to examine changes in the

emission spectrum associated with the binding of a particular concentration of inhibitor. The advantage to this approach is that the sample does not require constant rigorous stirring over a significant time period. This method is especially advantageous when analyzing the very slow binding of Type II/III inhibitors to kinases (Pargellis et al., 2002). A typical K_d determination is outlined below for each method.

4.2.1 Titration of ligand with the FLiK kinase (for rapidly binding ligands)

1. Prepare a cuvette containing a mini stir bar and 50–100 n*M* suspension of the labeled kinase. Place it into the fluorescence spectrometer and then measure the emission spectra for the labeled protein before adding any ligand.
2. Calculate and record the ratio of intensities at the two chosen emission wavelengths.
3. Using inhibitor stocks (prepared in DMSO), inject increasing amounts of compound into the stirring protein suspension. If possible, use concentrated stocks to minimize the required volume needed for each compound addition. Ideally, the total (%v/v) DMSO at the end of the titration should remain <1% to avoid undesirable effects on protein stability over time.
4. After each addition, allow sufficient time for the binding to reach completion (stable spectrum). For ligands which bind rapidly, stirring for 30 s should be sufficient to ensure that equilibrium has been reached. Measure the emission spectrum following each addition of ligand, calculate and record the emission ratio as in step no. 2.
5. Plot the data using desired graphing software. A plot of inhibitor concentration as a function of emission ratio will generate a saturable binding curve. Using a logarithmic plot of inhibitor concentration as a function of emission ratio values will generate a sigmoidal binding curve. The K_d value is obtained from the midpoint of the curve, which is the concentration of ligand required to occupy 50% of the FLiK kinase. The %binding to the kinase is a way to quantify the percent of acrylodan-labeled kinase in the well which has adopted the DFG-out conformation in response to the binding of a compound and provides a straightforward comparison to the positive control inhibitor. The %binding is calculated from the fluorescence ratio (R) using the following equation:

$$\%\ \text{Binding} = \left(\left(R_{\text{hit}} - R_{\text{neg}}\right)/\left(R_{\text{pos}} - R_{\text{neg}}\right)\right) \times 100. \qquad (6.2)$$

4.2.2 Titration of ligand with the FLiK kinase (for slow-binding ligands)

1. Prepare a series of cuvettes (one cuvette per inhibitor concentration) containing a mini stir bar and a 50–100 n*M* suspension of the labeled kinase.
2. Starting at low concentrations, add the appropriate amount of inhibitor to each cuvette in the series using the prepared stocks. Briefly mix each sample by placing the cuvette on a magnetic stir plate for 10 s to disperse the inhibitor and DMSO throughout the sample.
3. Place the cuvettes into the fluorescence spectrometer and measure the emission spectra for each sample. Measuring the cuvettes repeatedly over time may provide valuable information about the slow time scale of binding for these ligands and reveal the time dependence of K_d values. For a slow-binding DFG-out-binding ligand, K_d curves typically shift leftward over time. Additionally, these measurements allow an easy assessment of the effect that incubation time has on the assay window. When the assay window starts to decrease, this may be a sign that the protein is no longer stable.
4. Calculate and record the ratio of intensities for all samples as described in Section 4.2.1.
5. Plot the data as described in step no. 5 of Section 4.2.1 to obtain time-dependent K_d values.

4.3. Kinetic measurements

In addition to measuring K_d values, the FLiK assay also allows for the easy determination of kinetic rate constants for binding (k_{on}) and dissociation (k_{off}). Kinetic rate constants such as k_{on} and k_{off} provide information and insight into the affinity of compounds since they are related to the K_d ($K_d = k_{off}/k_{on}$). Typically, these parameters are determined using surface plasmon resonance to guide further medicinal chemistry optimization of compounds (de Kloe et al., 2010; Markgren et al., 2002), but in this case, the FLiK assay can also be used to characterize the kinetic components and contributions to measured affinities. These parameters can be obtained by monitoring changes in fluorescence in real-time at a single wavelength (Fig. 6.3A). The association rate constant, k_{on}, is the fastest observed binding rate (k_{obs}) for a ligand. The k_{on} can be derived from the linear fit of a plot of k_{obs} values obtained at several inhibitor concentrations. The linear range of this plot will result in a straight line with slope equal to k_{on} (Fig. 6.3B). Similar plots and experiments can be used to experimentally

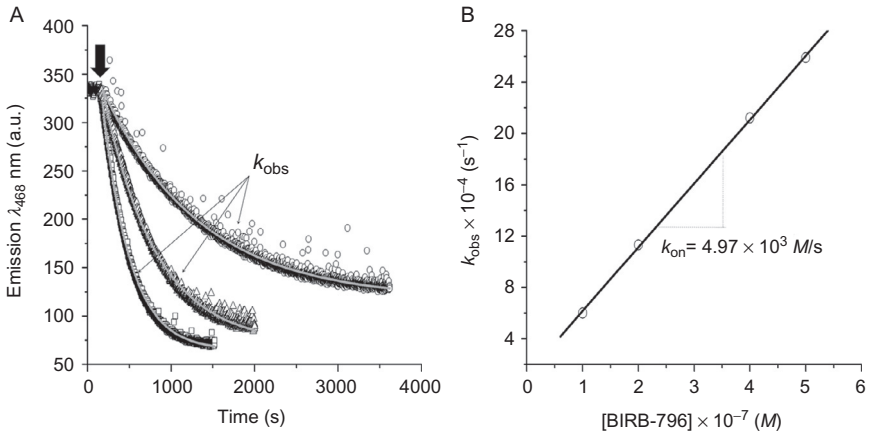

Figure 6.3 Determination of k_{on} for a DFG-out-binding ligand. This figure presents a typical set of kinetic-binding measurements for determining the k_{on} of BIRB-796 using acrylodan-labeled p38α. (A) The fluorescence emission is monitored at a single wavelength upon the addition of increasing concentrations of inhibitor. (B) These values can be plotted as a function of inhibitor concentration to obtain a linear curve with slope = k_{on}. *Panels have been reprinted with permission from Simard, Getlik, et al. (2009). Copyright 2009 American Chemical Society.*

derive the maximum k_{off} value for a ligand by adding increasing amounts of unlabeled kinase to a suspension of the fluorescent FLiK kinase prebound with different concentrations of inhibitor, thereby inducing dissociation of the ligand (Fig. 6.4). For slower binding ligands (>5 s), a standard fluorescence spectrometer is sufficient for resolving kinetic events. However, for rapid binders (<5 s), a stopped-flow apparatus might improve the time resolution of the measurement and enable accurate curve fitting. A typical k_{on} and k_{off} determination is outlined below for each method:

4.3.1 Determination of k_{on}

1. Prepare a cuvette containing a mini stir bar and a suspension of 50–100 n*M* kinase. Place it into the sample holder of the fluorescence spectrometer and set the excitation wavelength according to the fluorophore used to label the kinase.
2. Turn on the magnetic stirrer to begin mixing the sample. If temperatures above or below room temperature are required, the instrument should be equipped with temperature controls. Measured rate constants can be affected by sample temperature.

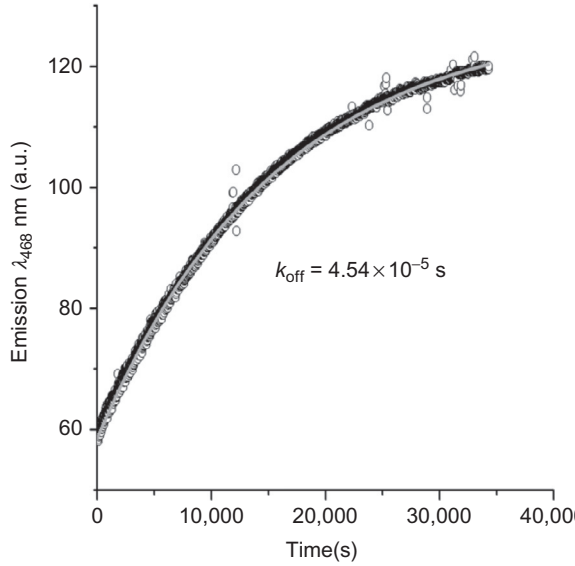

Figure 6.4 Determination of k_{off} for a DFG-out-binding ligand. This figure presents a typical kinetic measurement for determining the k_{off} of BIRB-796 using acrylodan-labeled p38α. The fluorescence emission is monitored at a single wavelength upon the addition of an excess of unlabeled kinase to scavenge the prebound ligand from the acrylodan-labeled kinase. *Panel has been reprinted with permission from Simard, Getlik, et al. (2009). Copyright 2009 American Chemical Society.*

3. Monitor the emission intensity at a wavelength, which is the most sensitive to ligand binding. This wavelength can be determined by performing the experiments described in Section 4.2.1.
4. Measure baseline fluorescence signal for ~30 s to ensure that the baseline is relatively flat and linear. Minor downward drift in the fluorescence signal over time can occur with certain fluorophores. However, the magnitude of this downward drift is often insignificant compared to the fluorescence changes induced by ligand binding.
5. Using the K_d curve, choose ligand concentrations which are in the linear range of the curve. Use these concentrations for examining the kinetic parameters.
6. Using a 1–10-μL pipettor, add the inhibitor to the stirring sample. For the best results, make sure the pipette tips are long enough to reach far into the cuvette to ensure that inhibitor is rapidly dispersed while mixing. To prevent outside light from entering the measurement chamber of the spectrometer, instruments which have an injection port

positioned above the cuvette are recommended. This allows ligand to be added while measuring fluorescence signal simultaneously.

7. Measure the change in emission over time; allow the fluorescence change to proceed until it linearizes. Repeat for at least four different ligand concentrations, using a new sample of protein for each measurement.
8. Plot the data using desired graphing software. Fit the kinetic trace using a first order fitting function to determine the observed rate constant (k_{obs}) for each dose of added ligand. If the graphing software provides the fit result as a half-time of fluorescence decay ($t_{1/2}$), the following equation should be applied to determine k_{obs}:$k_{obs} = \ln 2/t_{1/2}$. The units of $t_{1/2}$ should be converted to seconds prior to the conversion.
9. Plot k_{obs} (units of s) as a function of inhibitor concentration (units of M), fit the data using a linear fitting function. The slope of the resulting line is the true k_{on} (units of M/s) of the ligand. The k_{off} for the ligand can also be derived from the y-intercept of this plot (y-intercept $= k_{off}$).

4.3.2 Determination of k_{off}

1. Prepare a cuvette containing the FLiK kinase as described in step nos. 1 and 2 of Section 4.3.1.
2. Before measuring, add and preincubate the FLiK kinase with an inhibitor known to bind to a desired binding site and conformation of the kinase. Choose a concentration of ligand, which is in the linear range of K_d curve and allow the ligand to reach binding equilibrium.
3. Monitor fluorescence emission over time as described earlier in step no. 4 of Section 4.3.1.
4. Use a 1–10-μL pipettor to add a concentrated stock of unlabeled kinase to the sample, while measuring the change in emission over time until it becomes linear. Repeat by adding unlabeled kinase to a suspension of FLiK kinase preincubated with several concentrations of ligand.
5. Plot as the data using desired graphing software. Plot the kinetic trace and fit the curve as described above in step nos. 8 and 9 of Section 4.3.1 to determine the k_{off} for the ligand.

5. HTS WITH FLiK

5.1. Adaptation to HTS formats

The FLiK assay technology can easily be adapted to high-throughput assay plates (96-, 384-, and 1536-well formats). Adaptation of the assay to small

volume microtiter plates dramatically reduces the amount kinase required while increasing throughput to enable rapid screening of large compound libraries. The following steps should be taken for adapting the FLiK assay to HTS formats:

1. Prepare a suspension of 50 n*M* FLiK kinase and place equal volumes of sample into several wells of a black assay plate. Place the HTS plate into a fluorescence plate reader and set the excitation wavelength according to the fluorophore used to label the kinase.
2. Measure the emission spectrum of the kinase in the presence and absence of the positive control inhibitor to verify that the desired maxima have not shifted in the HTS format when compared to the cuvette format. For some kinases, we have observed as much as a 10 nm shift in one or both of the maxima when measured in HTS plates. If changes are observed, adjust the wavelengths used to calculate the ratio in HTS plates.
3. Calculate the Z' factor of the HTS assay using several wells containing positive and negative controls.
4. Assess protein stability and estimate the required incubation time for ligand binding to reach equilibrium. Prepare a row of wells containing a dilution series of the compound and a fixed concentration of the FLiK kinase. Measure the same plates over a period of time and monitor the leftward shift of the K_d-binding curve. Additionally, recalculate the Z' factor at each time point. Choose an incubation time, which produces both a stable K_d value and maximal assay window. A drop in the assay window or a decrease in data point reproducibility often indicates that the protein is no longer stable, resulting in lower calculated Z'-factor values. To minimize these effects during long incubation times, keep the plate sealed (in a humidity chamber or with adhesive seals) and protected from light.
5. If the K_d values and Z' factor need to be improved, several assay variables can be tested to examine their effects on the quality and reproducibility of the assay. These optimizations should be carried out for each FLiK assay. DMSO concentration (added with the compound), detergent type, detergent concentration, kinase concentration, volume in each well, plate type, incubation time, and incubation temperature are several examples. We have found that DMSO is critical to protein stability while detergent type and concentration can shift measured K_d values, depending on the assay. Detergent concentrations $<0.01\%$ (v/v) are recommended.

6. Using the optimized assay conditions, run a mock HTS scenario using liquid-handling robots to dispense kinase into two assay plates. Add compound to one plate (positive control) while adding only vehicle (buffer with DMSO) to the other plate. Incubate the plates and measure. Assess the full assay plates for good signal uniformity and calculate the Z' factor for the final optimized HTS assay.

5.2. HTS of compound libraries

We have performed several HTS using various FLiK kinases and found that 50–100 nM kinase is sufficient for avoiding many fluorescence artifacts and ensures that the signal from the fluorophore-labeled kinase is large enough to overcome the intrinsic fluorescence of many compounds when screened at a maximum concentration of 10–20 μM. A typical primary screen involves assay plates containing one compound per well at a maximum concentration of 10–20 μM. To assess background fluorescence of the compounds alone, each compound plate can be screened twice, once with only buffer and once with the FLiK kinase in the same buffer to enable background subtraction. Typically, compounds which bind at least 50% compared to the positive control at 10–20 μM are chosen for follow-up screening. After a primary screen, hits are selected and rescreened in a dose–response format to confirm binding to the kinase and to determine the K_d value. A general guideline for an HTS is provided below.

1. Map out the planned arrangement of a 384-well screening plate with the location of the compounds which will be added to the screening plate. Be sure to include several wells for both negative (DMSO) and positive (DFG-out inhibitor) controls.
2. Prepare all compound stocks (single point or dose–response) in 100% DMSO. Transfer compounds to a deep well assay plate and predilute in Assay Buffer. Place positive and negative controls into the appropriate wells of the predilution plate. This plate is the inhibitor stock plate for the assay. Ideally, the concentration of DMSO in the inhibitor stock plate should be 5% (v/v) or lower.
3. Use multichannel pipettors or liquid-handling devices to transfer compounds from the prediluted inhibitor stock plate to an empty small volume (20 μL), black, 384-well HTS plate.
4. Use multichannel pipettors or liquid-handling devices to transfer FLiK kinase in buffer to the assay plate. Addition of a larger volume of kinase solution should be sufficient to mix the contents of each well. The final

mixture of kinase and compound should be at 1 × in the assay plate and the final concentration of DMSO should ideally be 1% (v/v) or lower.

5. Seal and store the plates in darkness as described in step no. 4 of Section 5.1 using the previously determined incubation time and temperature.
6. After incubation, remove the seal from each plate and place the plate inside a microtiter plate reader to measure the fluorescence emission intensity at both desired wavelengths. Depending on the software which accompanies the instrument, the readout will either be the calculated ratio value or the two sets of individually measured emission intensities at each wavelength.
7. Identify compounds which trigger the expected fluorescence response and rescreen these compounds in a dose–response format using a similar procedure as described earlier for the primary screen.

5.3. Data analysis, fluorescence artifacts, and pitfalls

As is the case with any fluoresce-based assay, the identification of false positives can be tricky and sometimes difficult. Especially in the case of the FLiK assay, identifying such compounds can be very time consuming, especially since it is difficult to run all the necessary controls needed to identify all the possible types of fluorescence artifacts. The method described earlier, in which a background plate is used, will quickly identify intrinsic compound fluorescence which can be easily subtracted to reveal the emission signal contribution of the fluorophore label used in the FLiK kinase. In many cases, subtraction of the intrinsic signal from the compound reveals that the calculated ratio of emission intensities of the fluorophore is changing in a dose-dependent manner. To date, we have identified and confirmed the binding of several compounds, which also possess intrinsic background fluorescence.

The use of background plates will not account for other types of fluorescence artifacts such as quenching or synergizing interactions between the fluorophore and certain compounds. The artifacts are more difficult to identify and may not necessarily be observed if the compounds are placed into suspension with free, unreacted fluorophore since the fluorescence characteristics and behavior of reactive fluorophores often change upon conjugation to proteins. Once conjugated, the fluorescence emission of environmentally sensitive fluorophores depends on the local environment provided by the kinase conformation. By binding to the kinase, the ligands come into close proximity of the fluorophore and this close distance may

enable the observed quenching or synergizing interactions which would otherwise not occur with free fluorophore in solution. However, we should note that we have identified and confirmed the binding of some compounds, which also quench or augment the emission spectrum of the FLiK kinase (Simard, Grutter, et al., 2009). We have found that these types of compounds tend to change the overall intensity of the entire spectrum but also induce the expected change in the calculated ratio value in a dose-dependent manner. In other words, although the absolute intensity of the spectrum has been changed, the behavior of the fluorophore in response to binding has not been affected.

It is important to note that hit rates in the FLiK assay tend to be low due to the ability of the assay to report on ligands which only induce certain conformational changes. Although many more compounds may bind, only those inducing the desired conformational change will be selected for follow-up, making it possible to advance smaller collections of hits to subsequent screening in alternative assays. Rather than developing unique FLiK assays aimed at each type of fluorescence artifact, we instead use the multipronged approach described below to weed out and remove ligands remaining in this small collection of hits that may not truly bind or induce the desired conformational change.

1. Perform a primary screen as described in Section 5.2 and identify hits, which change the ratio of the FLiK kinase.
2. Prepare hits as a dilution series and retest them in a dose–response format in the FLiK assay using a background plate in addition to an assay plate containing the FLiK kinase. Incubate and then measure each plate (kinase plate and buffer plate) at the two chosen wavelengths. Calculate the ratio values for each compound concentration using values for each wavelength. Calculate the ratio for both the background corrected and uncorrected data. Compounds, which are not fluorescent, produce equivalent results regardless of whether background fluorescence is factored into the calculation. In contrast, fluorescent compounds produce very different results. These compounds should warrant closer analysis of the raw data before deciding if the compound is a valid hit. Typically, valid hits will produce a binding curve when screened in this format. For fluorescent compounds, the binding curve may only be visible following background subtraction at each concentration. For compounds, which are suspected of synergizing or quenching of the fluorophore, validation of binding can be more difficult. Such problematic compounds may be deemed less desirable for follow-up studies and removed from the

screen. However, a more conservative approach would include these compounds in follow-up studies with alternative assays.

3. Examine the binding kinetics of each hit in the FLiK assay. Fluorescence artifacts are less likely to show time dependence. This is especially useful when examining potential slow-binding hits identified with the DFG-out FLiK assay screen in which the kinase is labeled with a fluorophore on the activation loop.
4. Choose a hit list for subsequent testing in alternative binding assays for the kinase of interest, if available. Fluorescence polarization assays are an example. Displacement of a fluorescent prebound probe will change the polarization of the fluorophore and confirm that the hits are binding to the kinase.
5. Test the compounds in activity-based assays, if available, to confirm inhibition of catalytic activity. Since the phosphorylated kinase is often required for these types of assays, shifts in ligand potency may be observed if testing hits identified in a DFG-out FLiK assay in which the unphosphorylated kinase was used to enhance the likelihood of achieving the DFG-out conformation.
6. Attempt to crystallize hits, which show a positive response in all of the above assays. A crystal structure will reveal the details of the binding mode and validate the FLiK assay used to initially identify the compound.

6. SUMMARY

The poor selectivity of kinase inhibitors can be addressed by moving away from classical ATP-competitive inhibitors and targeting alternative kinase conformations and allosteric-binding sites. Profiling compounds against the entire kinome provides valuable information about the affinity and selectivity of compounds *in vitro*. However, optimizing compounds to lengthen drug–target residence times will ultimately provide a more complete context for fully understanding kinase inhibitor selectivity *in vivo*. By considering the kinetic components (k_{on} and k_{off}) of affinity, lead optimization strategies may improve *in vivo* selectivity and efficacy by directing medicinal chemistry efforts around improving the residence time of the ligand for the desired kinase.

We have developed FLiK as a HTS technology, which enables the rapid and robust identification of ligands, which bind to and stabilize specific kinase conformations. The FLiK approach allows straightforward determination of

ligand affinity (K_d values) and kinetic characterization (k_{on} and k_{off}) to better understand the kinetic factors, which contribute to the measured affinity. Moreover, FLiK does not require kinase activity or prior knowledge of the substrate, which may be advantageous when studying novel or less-characterized kinases. To date, the FLiK approach has been used to successfully monitor conformational changes in the activation loop of both Ser/Thr and Tyr kinases associated with the slow binding of DFG-out inhibitors. It has also been adapted to detect ATP-competitive inhibitors, which engage the glycine-rich loop (P loop) as well as Type IV ligands, which bind to remote allosteric-binding sites outside of the ATP-binding cleft.

ACKNOWLEDGMENTS

The German Federal Ministry for Education and Research supported this work through the German National Genome Research Network-Plus (NGFNPlus) (Grant No. BMBF 01GS08104). MSD, Bayer HealthCare, Merck-Serono, and Bayer CropScience are thanked for financial support.

REFERENCES

Akella, R., Moon, T. M., & Goldsmith, E. J. (2008). Unique MAP kinase binding sites. *Biochimica et Biophysica Acta*, *1784*, 48–55.

Anastassiadis, T., Deacon, S. W., Devarajan, K., Ma, H., & Peterson, J. R. (2011). Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. *Nature Biotechnology*, *29*, 1039–1045.

Arnold, K., Bordoli, L., Kopp, J., & Schwede, T. (2006). The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics*, *22*, 195–201.

Backes, A. C., Zech, B., Felber, B., Klebl, B., & Müller, G. (2008a). Small-molecule inhibitors binding to protein kinases. Part I: Exceptions from the traditional pharmacophore approach of type I inhibition. *Expert Opinion on Drug Discovery*, *3*, 1409–1425.

Backes, A. C., Zech, B., Felber, B., Klebl, B., & Müller, G. (2008b). Small-molecule inhibitors binding to protein kinase. Part II: The novel pharmacophore approach of type II and type III inhibition. *Expert Opinion on Drug Discovery*, *3*, 1427–1449.

Copeland, R. A. (2011). Conformational adaptation in drug–target interactions and residence time. *Future Medicinal Chemistry*, *3*, 1491–1501.

Copeland, R. A., Pompliano, D. L., & Meek, T. D. (2006). Drug–target residence time and its implications for lead optimization. *Nature Reviews. Drug Discovery*, *5*, 730–739.

Davis, M. I., Hunt, J. P., Herrgard, S., Ciceri, P., Wodicka, L. M., Pallares, G., et al. (2011). Comprehensive analysis of kinase inhibitor selectivity. *Nature Biotechnology*, *29*, 1046–1051.

de Kloe, G. E., Retra, K., Geitmann, M., Kallblad, P., Nahar, T., van Elk, R., et al. (2010). Surface plasmon resonance biosensor based fragment screening using acetylcholine binding protein identifies ligand efficiency hot spots (LE hot spots) by deconstruction of nicotinic acetylcholine receptor alpha7 ligands. *Journal of Medicinal Chemistry*, *53*, 7192–7201.

de Lorimier, R. M., Smith, J. J., Dwyer, M. A., Looger, L. L., Sali, K. M., Paavola, C. D., et al. (2002). Construction of a fluorescent biosensor family. *Protein Science*, *11*, 2655–2675.

DeLano, W. L. (2002). *The PyMOL molecular graphics system*. *http://www.pymol.org*.
Getlik, M., Simard, J. R., Termathe, M., Grutter, C., Rabiller, M., van Otterlo, W. A., et al. (2012). Fluorophore labeled kinase detects ligands that bind within the MAPK insert of p38alpha kinase. *PLoS One*, *7*, e39713.
Grutter, C., Simard, J. R., Mayer-Wrangowski, S. C., Schreier, P. H., Perez-Martin, J., Richters, A., et al. (2012). Targeting GSK3 from Ustilago maydis: Type-II kinase inhibitors as potential antifungals. *ACS Chemical Biology*, *7*, 1257–1267.
Karaman, M. W., Herrgard, S., Treiber, D. K., Gallant, P., Atteridge, C. E., Campbell, B. T., et al. (2008). A quantitative analysis of kinase inhibitor selectivity. *Nature Biotechnology*, *26*, 127–132.
Knight, Z. A., Lin, H., & Shokat, K. M. (2010). Targeting the cancer kinome through polypharmacology. *Nature Reviews. Cancer*, *10*, 130–137.
Lambert, C., Leonard, N., De Bolle, X., & Depiereux, E. (2002). ESyPred3D: Prediction of proteins 3D structures. *Bioinformatics*, *18*, 1250–1256.
Liu, Y., & Gray, N. S. (2006). Rational design of inhibitors that bind to inactive kinase conformations. *Nature Chemical Biology*, *2*, 358–364.
Markgren, P. O., Schaal, W., Hamalainen, M., Karlen, A., Hallberg, A., Samuelsson, B., et al. (2002). Relationships between structure and interaction kinetics for HIV-1 protease inhibitors. *Journal of Medicinal Chemistry*, *45*, 5430–5439.
Morphy, R. (2010). Selectively nonselective kinase inhibition: Striking the right balance. *Journal of Medicinal Chemistry*, *53*, 1413–1437.
Pargellis, C., Tong, L., Churchill, L., Cirillo, P. F., Gilmore, T., Graham, A. G., et al. (2002). Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nature Structural Biology*, *9*, 268–272.
Rabiller, M., Getlik, M., Kluter, S., Richters, A., Tuckmantel, S., Simard, J. R., et al. (2010). Proteus in the world of proteins: Conformational changes in protein kinases. *Archiv der Pharmazie (Weinheim)*, *343*, 193–206.
Richieri, G. V., Ogata, R. T., & Kleinfeld, A. M. (1999). The measurement of free fatty acid concentration with the fluorescent probe ADIFAB: A practical guide for the use of the ADIFAB probe. *Molecular and Cellular Biochemistry*, *192*, 87–94.
Schneider, R., Becker, C., Simard, J. R., Getlik, M., Bohlke, N., Janning, P., et al. (2012). Direct binding assay for the detection of type IV allosteric inhibitors of Abl. *Journal of the American Chemical Society*, *134*, 9138–9141.
Simard, J. R., Getlik, M., Grutter, C., Pawar, V., Wulfert, S., Rabiller, M., et al. (2009). Development of a fluorescent-tagged kinase assay system for the detection and characterization of allosteric kinase inhibitors. *Journal of the American Chemical Society*, *131*, 13286–13296.
Simard, J. R., Getlik, M., Grutter, C., Schneider, R., Wulfert, S., & Rauh, D. (2010). Fluorophore labeling of the glycine-rich loop as a method of identifying inhibitors that bind to active and inactive kinase conformations. *Journal of the American Chemical Society*, *132*, 4152–4160.
Simard, J. R., Grutter, C., Pawar, V., Aust, B., Wolf, A., Rabiller, M., et al. (2009). High-throughput screening to identify inhibitors which stabilize inactive kinase conformations in p38alpha. *Journal of the American Chemical Society*, *131*, 18478–18488.
Simard, J. R., Kluter, S., Grutter, C., Getlik, M., Rabiller, M., Rode, H. B., et al. (2009). A new screening assay for allosteric inhibitors of cSrc. *Nature Chemical Biology*, *5*, 394–396.