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Fluorophore Labeling of the Glycine-Rich Loop as a Method of Identifying Inhibitors That Bind to Active and Inactive Kinase Conformations†

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Abstract: Targeting protein kinases with small organic molecules is a promising strategy to regulate unwanted kinase activity in both chemical biology and medicinal chemistry research. Traditionally, kinase inhibitors are identified in activity-based screening assays using enzymatically active kinase preparations to measure the perturbation of substrate phosphorylation, often resulting in the enrichment of classical ATP competitive (Type I) inhibitors. However, addressing enzymatically incompetent kinase conformations offers new opportunities for targeted therapies and is moving to the forefront of kinase inhibitor research. Here we report the development of a new FLiK (Fluorescent Labels in Kinases) binding assay to detect small molecules that induce changes in the conformation of the glycine-rich loop. Due to cross-talk between the glycine-rich loop and the activation loop in kinases, this alternative labeling approach can also detect ligands that stabilize inactive kinase conformations, including slow-binding Type II and Type III kinase inhibitors. Protein X-ray crystallography validated the assay results and identified a novel DFG-out binding mode for a quinazoline-based inhibitor in p38α kinase. We also detected the high-affinity binding of a clinically relevant and specific VEGFR2 inhibitor, and we provide structural details of its binding mode in p38α, in which it stabilizes the DFG-out conformation. Last, we demonstrate the power of this new FLiK labeling strategy to detect the binding of Type I ligands that induce conformational changes in the glycine-rich loop as a means of gaining affinity for the target kinase. This approach may be a useful alternative to develop direct binding assays for kinases that do not adopt the DFG-out conformation while also avoiding the use of expensive kits, detection reagents, or radioactivity frequently employed with activity-based assays.

1. Introduction

Protein kinases are an important set of enzymes regulating key cellular processes. The improved understanding of aberrantly regulated kinase signaling in cancer biology[1] has initiated the area of targeted tumor therapy and led to the development of small organic molecules that specifically target unwanted kinase activities.[2] Most small organic molecules modulating kinase activity are Type I inhibitors such as staurosporine and dasatinib, which typically inhibit a kinase by binding to the active "DFG-in" conformation. In this conformation, the regulatory activation loop is open and extended, which allows ATP and substrates to bind.[3] The adenine of ATP forms a crucial hydrogen bond with the hinge region of the kinase—a short, flexible region located between the N- and C-terminal lobes of the kinase domain—while the β and γ phosphates of ATP are coordinated by a complex network of ionic and hydrogen-bonding interactions with several structural elements, including Mg^{2+} or Mn^{2+} ions, the Asp side chain of the conserved DFG motif, and amino acid residues in the glycine-rich loop located above the ATP binding cleft.[4] Most Type I inhibitors also form hydrogen bond(s) with the hinge region of the kinase and thereby compete with the binding of ATP. Given the highly conserved nature of the ATP binding pocket, poor Type I inhibitor selectivity and efficacy is a bottleneck in the development of effective long-term cancer treatments.[5] Modern kinase inhibitor research is therefore currently shifting focus to the identification of new chemical entities that bind to and stabilize the enzymatically incompetent "DFG-out" kinase conformation.[2] The DFG-out conformation results from structural changes in the activation loop induced by a flip of the highly conserved DFG motif,[6,7] an event that also exposes a less-conserved "allosteric" site adjacent to the ATP binding site. Studies into the druggability of this site are moving to the forefront of kinase inhibitor research.[8] For example, Type II inhibitors such as sorafenib (Nexavar)[9] and imatinib (Gleevec)[10] bind to the hinge

† All crystal structures described in this paper were deposited into the Protein Data Bank (PDB codes: 3HUC, 3HUB, 3L8S).

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region and are ATP-competitive but also extend deep into this allosteric site, while Type III inhibitors bind exclusively within the allosteric pocket.[7,11]

Although molecules that utilize this less-conserved site are believed to have superior selectivity profiles and improved pharmacological properties and may offer new opportunities for drug development,[6,12] approaches that could discriminate between different inhibitor types and identify inhibitors that stabilize the inactive DFG-out conformation were not compatible with high-throughput screening (HTS) formats used by academia and industry.[13] We previously developed a robust new binding assay system (FLiK, Fluorescent Labels in Kinases), in which we tagged the activation loop of cSrc and p38α kinases with acrylodan,[11,14] allowing for the direct measurement of the dissociation constant (K_d), rate constant of association (k_{on}), and rate constant of dissociation (k_{off}) of various ligands. More recently, two additional binding assays based on the displacement of prebound probes from p38α kinase were also reported: one made use of a fluorophore-labeled inhibitor,[15] and the other employed an enzyme fragment complementation-based approach.[16] In the latter case, a chemiluminescence read-out was generated by the displacement of a prebound inhibitor−peptide probe, which then complements and activates β-galactosidase to catalyze a chemiluminescence reaction that serves as the assay read-out. Although these approaches were demonstrated to be suitable for determining the affinities of displacing ligands using end point measurements, analysis of kinetic parameters (k_{on} and k_{off}) is less straightforward since signal detection is rate-limited by the well-characterized slow dissociation of the chosen pyrazolourea-based probes from p38α.[7]

The FLiK approach has so far led to the identification of the first reported Type III ligands of cSrc,[11] identified a new Type III binding mode for the thiazole-urea scaffold in p38α, and also led to the identification of several unique Type I ligands that stabilize the DFG-out conformation of p38α. The sensitivity in detecting ligands that stabilize the DFG-out conformation is significantly enhanced by using the FLiK approach to screen compound libraries since it utilizes the unphosphorylated inactive form of the kinase. However, manipulation of the activation loop by mutation and subsequent fluorophore labeling may not be tolerated by some kinases, may significantly alter the DFG-in/out equilibrium, and could lead to significant changes in the affinity of known inhibitors of the target kinase. Moreover, there is still no method available for predicting whether a kinase of interest can even adopt the DFG-out conformation. Therefore, it would be useful to develop alternative labeling strategies for sensitively detecting DFG-out binders as well as ligands that may bind in the ATP site and induce other conformational changes in target kinases not regulated by the DFG-in/out switch, thereby making the FLiK approach applicable to a larger number of kinases.

Here we report on the development of a fluorescent-labeled kinase assay system that takes advantage of cross-talk between the activation loop and the glycine-rich loop of the kinase domain (Figure 1). Aside from the activation loop, the glycine-rich loop (or P-loop) is another highly conserved flexible structural element located in the N-terminal lobe of kinases and contains the canonical Gly-X-Gly-X-X-Gly motif (where X may be any amino acid). Through the formation of a hydrophobic interface, it is believed that these two loops interact with one another (Figure 1a,b) and modulate the conformational equilibrium of the kinase.[9] This cross-talk is also partly mediated by the nearby helix C in many kinases, which forms the "roof" of the allosteric site available in the DFG-out conformation.[17] Thus, the glycine-rich loop also serves as an important determinant for inhibitor selectivity and affinity and responds to movements in the activation loop induced by Type II and Type III ligand binding within the allosteric site. Additionally, the glycine-rich loop serves as a regulatory "flap" that controls the entry of ATP and substrates into the ATP binding site[18] and has been shown to change conformation with the binding of some Type I inhibitors in the ATP binding pocket, thereby increasing their affinity by shielding them from the surrounding solvent.[19,20]

By labeling the glycine-rich loop of the serine-threonine kinase p38α with acrylodan at a specific position within the Gly-X-Gly-X-X-Gly motif, we were able to develop a kinase biosensor that can detect the binding of inhibitors with different binding modes. Type II and Type III inhibitors, which mediate a conformational change in the glycine-rich loop via movement of the activation loop to the DFG-out conformation, were easily discriminated in HTS formats by monitoring time-dependent changes in fluorescence signal or K_d over time, or in cuvettes by measuring k_{on} (<5 s for Type I binders). In addition to achieving our primary goal of detecting Type II and Type III inhibitors, this binding approach also sensitively detected Type I ligands, which gain affinity by interacting directly with the glycine-rich loop and, in some cases, also stabilize the DFG-out conformation. We present several crystal structures of compounds in complex with p38α and report a new binding mode for quinazoline-based inhibitors identified solely with this second-generation FLiK approach. Such information may prove to be useful in further design and synthesis efforts to redirect known scaffolds to inhibit inactive kinase conformations. We propose this new FLiK approach as a useful method for identifying new ligands for kinases that are not known to be regulated by the DFG-in/-out equilibrium, a required feature for the success of our original FLiK approach of labeling the activation loop of kinases.[11,14]

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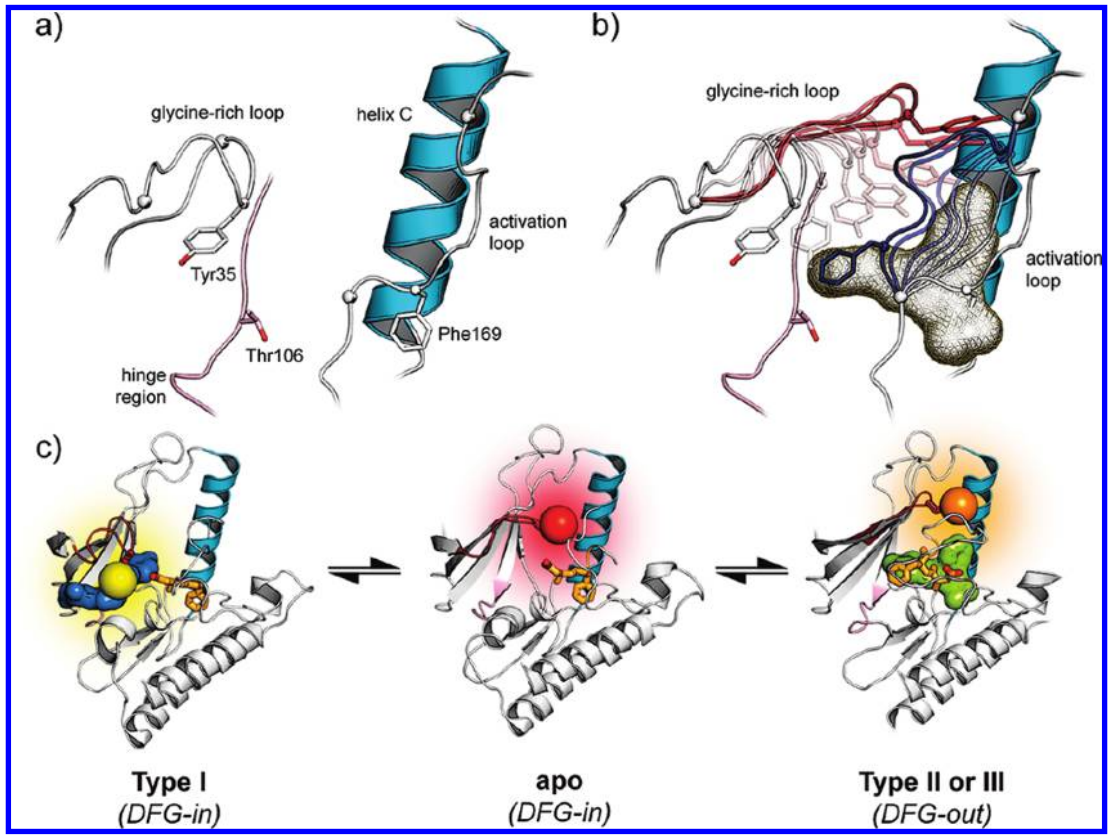

Figure 1. Conformational changes triggered by ligand binding in p38α. (a) Structurally important regions of the active (DFG-in) kinase domain are highlighted (helix C, light blue; hinge region, pink). (b) Type II/III inhibitor (mesh surface) binding to the DFG-out conformation causes a conformational change in the activation loop (white → dark blue) and allows the glycine-rich loop (white → dark red) to adopt a more extended conformation. (c) The glycine-rich loop (red) was labeled with a fluorophore (colored sphere) to generate a direct binding assay that detects conformational changes in this structural element. Some Type I inhibitors (blue surface) bind to the active kinase conformation (left panel) and directly interact with the glycine-rich loop. The glycine-rich loop adopts a more typical extended conformation in the absence of ligand (middle panel). Type II/III inhibitors (green surface) bind to inactive kinase conformations in which the glycine-rich loop adopts its most extended conformation.

2. Materials and Methods

2.1. Materials. The fluorophore 6-acryloyl-2-dimethylaminonaphthalene (acrylodan) was purchased from Invitrogen GmbH (Germany). Crystallization plates (EasyXtal Tool; 24-well) were obtained from Qiagen GmbH (Germany). Cuvettes and mini stir bars were obtained from Carl Roth GmbH (Germany). Small volume (20 μL fill volume) black flat bottom 384-well plates were obtained from Greiner Bio-One GmbH (Solingen, Germany). All supplies for the HTRF assay kit for p38α were purchased from CisBio (Bagnols-sur-Cèze, France).

2.2. Expression, Purification, and Labeling of p38α Kinase. The genes for all variants of p38α used in the described FLiK approach were synthesized containing the necessary mutations for specific acrylodan labeling on the glycine-rich loop by Geneart (Regensburg, Germany). The mutated human p38α kinase construct (Cys119Ser/Cys162Ser/Tyr35Cys) as well as wild-type p38α were subsequently subcloned into a pOPINF vector and transformed as an N-terminal His-tag construct with a PreScission Protease cleavage site into BL21(DE3) Codon+RIL *E. coli*. Protein expression, purification, fluorophore labeling, and verification of monolabeling of the kinase were performed essentially as described previously.[14]

Briefly, fluorophore labeling of both proteins was carried out by combining mutant p38α and the thiol-reactive fluorophore acrylodan (dissolved in DMSO) in 50 mM HEPES buffer (pH 7.0) at a molar ratio of 1:1.5, respectively, and allowed to react in the dark overnight at 4 °C. The conjugated protein was subsequently concentrated, washed three times in a 10k-MWCO Centricon using an appropriate storage buffer, aliquoted and frozen at −80 °C.

2.3. Analysis of p38α Labeling by HPLC and Mass Spectrometry. Monolabeling of the protein was verified by ESI-MS as described previously.[11,14] Purified unlabeled and acrylodan-labeled human p38α (∼1.5 mg/mL) were desalted using Protein Desalting Spin Columns (Pierce) according to the manufacturer's instructions. The protein was further prepared for analysis by adding up to 10% v/v acetonitrile. Aliquots (20 μL) were analyzed by ESI-MS using a Finnigan LCQ Advantage Max mass spectrometer from Thermo. Deconvolution and visualization were performed using the BioWorks 3.1 software from Thermo Scientific.

To assess irreversible labeling of the protein by the electrophile of the "hit" compound **6**, the unlabeled human p38α (∼1.5 mg/mL) was incubated with a 3:1 molar ratio of **6** for 20−30 min at room temperature and then desalted and analyzed using the procedure described above.

2.4. Fluorescence Characterization of Acrylodan-Labeled (Glycine-Rich Loop) p38α. Characterization of the fluorescence response of glycine-rich loop-labeled p38α was carried out using several types of inhibitors expected to interact with the glycine-rich loop either directly or indirectly (via movement of the activation loop). Acrylodan-labeled p38α was excited at 386 nm with excitation and emission slits set to 3 and 5 nm, respectively. Emission spectra were recorded every 30 s in the absence and in the presence of saturating concentrations of the DFG-out binding ligand, BIRB-796. These measurements revealed the most sensitive wavelength to monitor fluorescence changes (kinetics) in real-time (475 nm) and to assess the feasibility of using ratiometric fluorescence to plot binding curves (K_d). The emission spectra of glycine-rich loop-labeled p38α (unbound and DFG-out saturated)

were used to calculate the fluorescence parameter ΔR_{max} as described elsewhere[21] and revealed that the ratio of emission intensities at 475 and 512 nm ($R = I_{\lambda 512}/I_{\lambda 475}$) allowed the most sensitive detection of ligands that either directly or indirectly displace the glycine-rich loop upon ligand binding. In the case of p38α and BIRB-796, we calculated an average ΔR_{max} value of 0.303 ± 0.083.

2.5. End Point and Kinetic Measurements. To characterize this new labeling strategy, p38α labeled with acrylodan at the glycine-rich loop (50 nM) was screened against a small subset (~400) of compounds based on scaffolds that are generally known to be privileged for binding to the DFG-in or DFG-out conformation of kinases. The kinase was pre-incubated with various concentrations of inhibitor before end point fluorescence measurements were carried out in either polystyrene cuvettes or 384-well plates to determine the K_d of each compound. A standard buffer (50 mM Hepes, 200 mM NaCl, pH 7.45) was used for all experiments. For cuvette measurements, incubations were carried out overnight in the dark at 4 °C for p38α. For HTS formats, incubations were carried out for up to 5 h at room temperature. Long incubation times are needed to account for the time-dependence of Type II inhibitor binding to p38α.[7]

In the cuvette format, a series of cuvettes containing different amounts of inhibitor were prepared using inhibitor stocks (0.01, 0.1, 1.0, and 10.0 mM in DMSO). All measurements of the cuvettes were made with a JASCO FP-6500 fluorescence spectrophotometer (JASCO GmbH, Gross-Umstadt, Germany). A Tecan Safire^{II} (Tecan Deutschland GmbH, Germany) was used to measure the fluorescence read-out in the 384-well plate format. The % v/v DMSO did not exceed 0.2% in cuvettes and was 5% v/v in 384-well plates. In the case of p38α, average Z' factors of 0.67 ± 0.05 ($n = 6$) and 0.64 ± 0.10 ($n = 6$) were determined for the cuvette and 384-well formats, respectively, using saturating amounts of BIRB-796 or sorafenib[14] as a positive control for a ligand that induces glycine-rich loop movement. Vehicle (DMSO) was used as the negative control.

For acrylodan-labeled p38α, ratiometric fluorescence values ($R = I_{\lambda 512}/I_{\lambda 475}$) enabled reliable binding curves of detected compounds to be plotted, allowing for direct determination of the K_d of ligand binding. It should be noted that binding modes of detected molecules can initially be assessed in HTS formats by measuring plates from primary and/or secondary screens at different time points. Compounds that change the maximum ratiometric signal or K_d over time are likely to be Type II/III inhibitors. Alternatively, kinetics of the association (k_{on}) and dissociation (k_{off}) of selected compounds can be determined using cuvettes as described previously.[14] To determine k_{on}, a mini stir bar was placed in the bottom of each cuvette to ensure rapid mixing as inhibitor was delivered through the injection port located above the cuvette. Fluorescence changes were monitored at 475 nm for p38α in real-time using a JASCO FP-6500 fluorescence spectrophotometer (JASCO GmbH, Groβ-Umstadt, Germany). Nearly instantaneous binding kinetics (<5 s) are characteristic of Type I inhibitors, while slower kinetics (>10 s) indicate the slow binding of Type II or Type III inhibitors to the DFG-out conformation. Following binding, k_{off} was determined by adding a 10-fold excess of unlabeled kinase to shift the binding equilibrium away from the labeled kinase, as described previously.[14] Addition of excess unlabeled kinase causes the inhibitor to redistribute and dissociate from acrylodan-labeled p38α, resulting in a recovery of the fluorescence signal. All binding and dissociation curves were fit to a single exponential equation: $F(t) = F(\infty) + F(0)\exp(-tk_{obs})$, where t is time, $F(0)$ is the initial fluorescence intensity, and $F(\infty)$ is the fluorescence at $t = \infty$. The half-time of fluorescence decay ($t_{1/2}$) was calculated with the following equation: $t_{1/2} = \ln 2/k_{obs}$.

2.6. Co-crystallizations of p38α with Detected Ligands. Inhibitors were co-crystallized with unlabeled p38α using conditions previously reported.[14,22] Briefly, protein−inhibitor complexes were prepared by mixing 30 μL of p38α (10 mg/mL) with 0.3 μL of inhibitor (100 mM in DMSO) and incubating the mixture for 1−2 h on ice. Samples were centrifuged at 13 000 rpm for 5 min to remove excess inhibitor. Crystals were grown in 24-well crystallization plates using the hanging drop vapor diffusion method and by mixing 1.5 μL of protein−inhibitor solution with 0.5 μL of reservoir (100 mM MES pH 5.6−6.2, 20 − 30% PEG4000 and 50 mM *n*-octyl-β-D-glucopyranoside).

3. Results

3.1. Selection of the Labeling Position on the Glycine-Rich Loop. The serine/threonine kinase p38α is an attractive drug target and has been extensively characterized for drug design. Additionally, the dynamics of protein−ligand interactions as well as p38α conformational dynamics have been studied using both protein X-ray crystallography and NMR approaches and have provided insights into cross-talk between the activation loop and the glycine-rich loop.[23,24] These data guided us to use p38α as the model kinase for development and characterization of an alternative FLiK approach that involves a glycine-rich loop labeling strategy to detect and characterize the binding of DFG-out binding Type II and III inhibitors.

We replaced surface-exposed cysteines with serines and introduced a new Cys residue (Tyr35Cys) via site-directed mutagenesis into the glycine-rich loop of p38α at the position that directly precedes the third Gly of the Gly-X-Gly-X-X*-Gly motif (as indicated by *). Single labeling of the kinase with the environmentally sensitive fluorophore acrylodan was carried out and verified by ESI-MS as described previously[14] (Figure S1, Supporting Information). The amino acid residue at the selected labeling position is conserved as a Tyr or Phe in ~80% of human kinases (Figure S2, Supporting Information), suggesting that labeling at this position may make this new FLiK approach applicable to a larger number of kinases. Furthermore, replacing a tyrosine with the comparably sized acrylodan-labeled cysteine should be well tolerated by the kinase. This was confirmed by using an activity-based assay to measure and compare the ATP-K_m of each phosphorylated active p38α variant (mutated/unlabeled and acrylodan-labeled) to that of wild-type p38α (Table S1, Supporting Information). Likewise, as observed previously for the activation loop-labeled p38α,[14] we observed no significant changes in the IC_{50} values of three known p38α inhibitors upon mutation or labeling of the glycine-rich loop, demonstrating that both FLiK assay approaches ultimately provided similar affinity data for detected ligands. Nevertheless, ATP-K_m data reported here and previously[14] suggest that the acrylodan labeling of the glycine-rich loop was better tolerated by p38α than labeling of the activation loop.

3.2. End Point and Real-Time Measurements: Determination of K_d, k_{on}, and k_{off}. To compare this approach with our previously reported method in which we labeled the activation loop of p38α, and to demonstrate that labeling the glycine-rich

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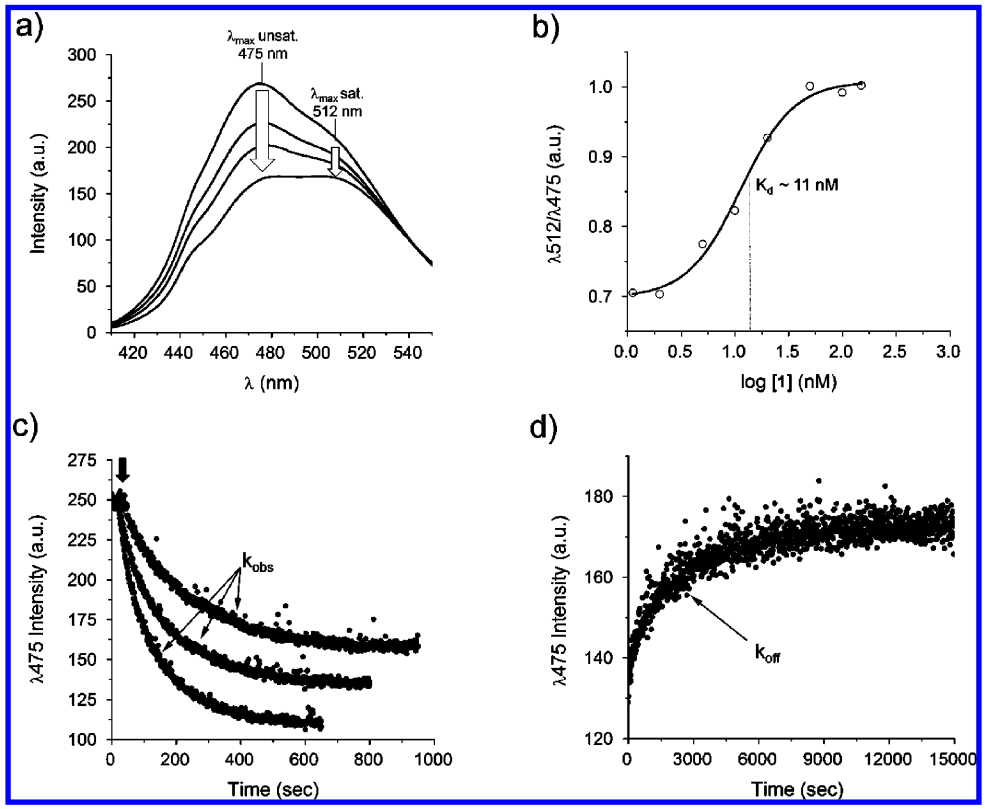

Figure 2. Fluorescence measurements using glycine-rich loop-labeled p38α incubated with **1** (BIRB-796). (a) Acrylodan emission at 475 nm decreases significantly relative to the emission at 512 nm (as indicated by arrows) in the presence of increasing amounts of the Type II p38α inhibitor **1**. (b) End point equilibrium measurements of ratiometric fluorescence ($R = I_{\lambda 512}/I_{\lambda 475}$) were made at several inhibitor concentrations to directly obtain the K_d (~11 nM for **1**). (c) Real-time fluorescence changes at 475 nm resulting from the addition (arrow) of different amounts of **1** were fit to a first-order decay function to obtain the observed rate constant (k_{obs}) for the binding of each dose of added inhibitor. The fluorescence decrease was dose-dependent. (d) Extraction of prebound **1** from glycine-rich loop-labeled p38α using an excess of unlabeled p38α allows direct determination of the dissociation rate constant (k_{off}). All experiments were carried out using 100 nM of the fluorescent-labeled kinase. End point and real-time kinetic data are representative of a typical single experiment for **1**.

loop with acrylodan serves as a reliable alternative approach, we used the Type II p38α inhibitor **1** (BIRB-796) to characterize the fluorescence response. End point measurements were carried out by measuring the emission spectrum of glycine-rich loop-labeled p38α in the presence of increasing concentrations of **1** (Figure 2a). Subsequently, a ratiometric fluorescence value ($R = I_{\lambda 512}/I_{\lambda 475}$) was plotted on a logarithmic scale against the concentration of ligand to directly determine $K_d = 9.5 \pm 2.7$ nM for **1** (Figure 2b), which is similar to our previously reported value of 7.5 ± 2.3 nM obtained with p38α labeled at the activation loop and demonstrates the reliability of K_d values for DFG-out binders obtained with this alternative approach.

A significant change of emission at 475 nm also made it possible to study the kinetics of dissociation and association in real-time for different concentrations of ligand. Upon addition of **1**, the fluorescence emission at 475 nm decreased in a dose-dependent manner and was nicely fit to a first-order kinetic (Figure 2c) to obtain the observed rate constant (k_{obs}) of binding for each dose of added ligand. These values can subsequently be plotted and fit linearly to obtain the true k_{on} for a ligand, which we determined to be $\sim 4.0 \times 10^4$ M^{-1} s^{-1} for **1**. Alternatively, addition of a 10-fold molar excess of unlabeled p38α to glycine-rich loop-labeled p38α prebound with **1** resulted in a first-order increase in fluorescence as the ligand dissociates from the labeled kinase, allowing direct determination of k_{off} (Figure 2d). These measurements demonstrate both the reversibility of the fluorescence response and the changing equilibrium that exists between the DFG-in and DFG-out conformations.

Determination of k_{on} and k_{off} also allows for an alternative route for calculating, rather than directly measuring, K_d values ($K_d = k_{off}/k_{on}$) and sheds light on the factors contributing to different ligand affinities. The values for k_{on} and k_{off} obtained here are comparable to those published elsewhere for **1**, found using alternative methods.[7] Furthermore, the obtained rate constants can be used to calculate $K_d = 7.1$ nM for **1**, which is similar to the calculated K_d previously reported, found by measuring k_{on} and k_{off} obtained with p38α labeled on the activation loop (11.9 nM[14]), again demonstrating that the glycine-rich loop labeling strategy is a reliable alternative approach to labeling the activation loop for the detection of DFG-out binding ligands.

3.3. Adaptation of Acrylodan-p38α to HTS Formats, Hit Identification, and Characterization. After the fluorescence response of glycine-rich loop-labeled p38α was characterized, the assay was adapted to HTS formats aimed at the sensitive detection of Type II and Type III inhibitors in a small subset of compounds available in our laboratory[16,25−28] comprising various scaffolds known to be privileged for binding to the DFG-in or DFG-out conformation of kinases. After an initial prescreen

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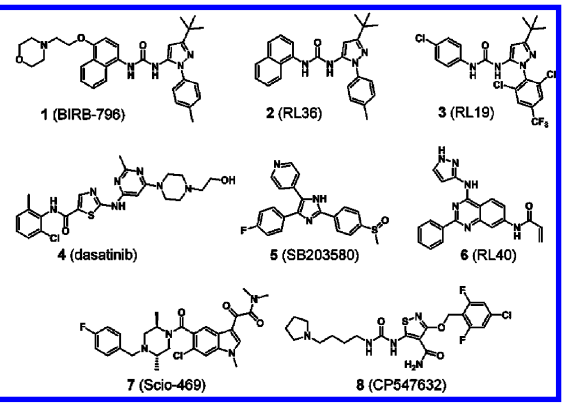
Table 1. Characterization of Inhibitor Types Using the Glycine-Rich Loop-Labeled Assay for p38α[a]

Cpd	Inhibitor Type	K_d (nM)	100 nM single dose: Binding $t_{1/2}$ (sec)	100 nM single dose: Dissociation $t_{1/2}$ (sec)
1	II	9.5 ± 2.7	~150	~2440
2	III	15.2 ± 0.8	~150	~1580
3	III	ND	ND	ND
4	I	ND	ND	ND
5	I	16.8 ± 4.2	< 2	< 10
6	I	1030 ± 180	< 2	< 10
7	I	8.2 ± 2.9	< 2	< 10
8	I	99.0 ± 13.7	< 2	< 10



[a] Compounds **3** and **4** were not detected (ND). **3** is a sterically bulky derivative of the classical pyrazolourea Type III inhibitors of p38α[7,29] that does not bind in the described allosteric site of p38α[14] and serves as a negative control for stabilization of the DFG-out conformation. **4** (dasatinib) is a known Type I inhibitor of Abl/cSrc and p38α[30] that binds to the active DFG-in conformation and does not interact with the glycine-rich loop and thus served as a negative response control for Type I inhibitors. All K_d determinations are the mean ± SD of at least three independent measurements.

at three fixed inhibitor concentrations (0.5, 5, and 50 μM), selected compounds, some of which were derivatives of known p38α inhibitors, were subjected to further studies using concentration-dependent direct binding measurements to determine k_{on}, k_{off}, and K_d (Table 1) (Figure S3, Supporting Information) and to further compare affinities of compounds detected with this new FLiK approach and the originally published approach[14] as a means of validation.

As expected, the known DFG-out binder **1** showed a distinct time dependence (Figure S4, Supporting Information) over a period of 5 h and was found to have a K_d value similar to that obtained previously using activation loop-labeled p38α.[14] Additionally, **1** and **2** were found to differ mainly with respect to k_{off} rather than k_{on} as the primary determinant for affinity, which is a well-characterized observation for the binding of pyrazolo-urea-based compounds to the DFG-out conformation of p38α.[7,14] The known Type I p38α inhibitor **5** was also sensitively detected and had a K_d value comparable to that published previously.[14] We previously reported that, despite adopting a Type I binding mode and contacting the kinase hinge region, **5** binds to p38α and can stabilize the DFG-out conformation by forming $\pi-\pi$ stacking interactions between the DFG Phe169 and Tyr35 of the glycine-rich loop,[14] thus explaining the sensitive detection of this compound using both FLiK approaches. The Type I binding mode of **5** was easily discriminated from Type II/III binders in a HTS format due to its more rapid binding and dissociation kinetics and because it did not show the same K_d time dependence as the DFG-out binder **1** (Figure S4). Thus, the FLiK assay also allows easy preliminary assessments of inhibitor binding mode without requiring co-crystallization of detected ligands with the protein.

Dasatinib (**4**), a Type I inhibitor that binds to p38α with a reported K_d = 27 nM,[30] was not detected using glycine-rich loop-labeled p38α, suggesting that it adopts the expected/published binding mode observed in the DFG-in conformation of Abl (PDB code: 2GQG) and cSrc (PDB code: 3G5D). However, it was surprising to observe that the indole derivative **7** and the 2-phenyl-substituted quinazoline **6**, both known to adopt the classical Type I binding mode and to contact the hinge region,[31,32] were detected using this approach. In the case of **7**, we determined $K_d = 8.2 \pm 2.9$ nM, which strongly agrees with the reported IC_{50} for this compound (~9 nM)[31] and demonstrates that our alternative FLiK approach is extremely sensitive to this ligand. Additionally, the VEGFR2 inhibitor **8** (CP-547632) was also detected and was found to have $K_d = 99 \pm 13$ nM. This compound is in clinical trials as an anticancer agent that acts by inhibiting angiogenesis and tumor growth mediated by VEGFR2.[33] Although no crystal structure for **8** in complex with a kinase has been published to date, previously reported pharmacokinetic studies revealed that **8** inhibits VEGFR2 in an ATP-competitive manner.[33] Real-time kinetic measurements of **6**, **7**, and **8** show rapid binding (<2 s) of all three compounds to p38α, which is consistent with the expected Type I binding mode. However, only **8** could be characterized using p38α labeled at either the activation loop or the glycine-rich loop; only the latter detected **6** and **7**. This suggests that our new approach has the added advantage of detecting certain Type I inhibitors that may induce unique conformational changes, most likely by making additional contacts to the acrylodan-labeled glycine-rich loop and altering its conformation.

To determine whether detection of these compounds was the result of direct interactions with the glycine-rich loop, binding to the DFG-out conformation, or both, we co-crystallized **6**, **7**, and **8** in complex with p38α and solved the structures to resolutions of 1.8, 2.25, and 2.35 Å, respectively. In the p38α−**7** complex (Figure 3A), the inhibitor resides in a typical Type I binding orientation and forms hydrogen bonds to the hinge region, analogous to those previously reported for a close structural analogue (PDB code: 2QD9). The fluorophenyl moiety of **7** extends beyond the gatekeeper residue and occupies the hydrophobic subpocket, an interaction that is known to increase the affinity of compounds for p38α.[34] Although the DFG motif is found in the active "in" conformation, the conformation of the glycine-rich loop is significantly altered in the case of p38α

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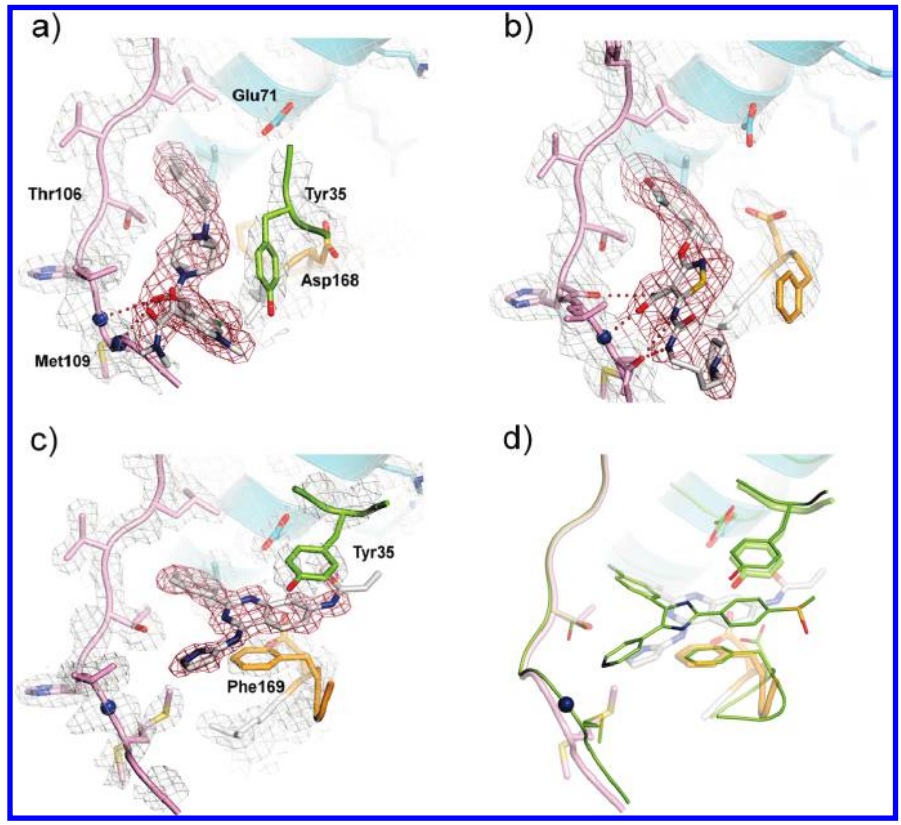

Figure 3. Crystal structures of wild-type p38α in complex with **6**, **7**, and **8**. Electron density maps ($2F_o - F_c$) of p38α (gray) and each ligand (red) are contoured at 1σ. (a) The carbonyl attached to the piperazine ring of **7** forms two hydrogen bonds (dashed red lines) to the hinge region (pink) (backbone NH of Met109 and Gly110). The glycine-rich loop (green) folds over to directly interact with the inhibitor and shields the indole moiety and piperazine ring from the solvent. The DFG motif (orange) is in the "in" conformation, with Asp168 pointing into the ATP binding site. (b) Similar to **7**, the halogen-substituted methoxybenzene of **8** bends around the gatekeeper (Thr106) and points into the hydrophobic subpocket. The carboxamide and the urea attached to the isothiazole ring both form hydrogen bonds to the hinge region (backbone CO of His107, NH and CO of Met109). The pyrrolidine−butane moiety is kinked by 90° and points away from the solvent into the ATP pocket. The glycine-rich loop is less visible in the electron density, and the DFG motif is clearly in the "out" conformation. (c) **6** is sandwiched between the side chains of Tyr35 of the glycine-rich loop (green) and Phe169 of the DFG motif (orange) and does not form any contacts to the hinge region (pink). The electron density as well as ESI-MS measurements proved that the electrophile at the 7-position of **6** does not react with the protein (see Figure S1, Supporting Information). (d) Structural alignment of the p38α−**6** complex and **5** (SB203580) bound to p38α (PDB code: 3GCP).[14] In the p38α−**5** complex (green), the pyridine ring of the inhibitor forms an essential hydrogen bond to the hinge region (Met109) of the kinase. The proximal phenyl substituent of **5** and the quinazoline core of **6** are isostructurally sandwiched between the glycine-rich loop and the DFG motif to form energetically favorable $\pi-\pi$ interactions with the side chains of Tyr35 and Phe169. In both structures, these interactions most likely stabilize the DFG motif in the "out" conformation.

bound with **7**, thus explaining its sensitive detection using only this new FLiK approach. The glycine-rich loop is folded over the inhibitor such that the side chain of Tyr35 partly shields the methyl-substituted piperazine ring and the chloro-substituted indole ring of **7** from the solvent, thereby stabilizing inhibitor binding, most likely via hydrophobic interactions. As a consequence, the removal of ordered water molecules from this surface of the ligand results in the high-affinity binding of **7** to p38α.

The crystal structure of **8** in complex with p38α (Figure 3b) revealed that the kinase was stabilized in the DFG-out conformation with the inhibitor bound also to the hinge region (Type I). The carboxamide attached to the isothiazole of the inhibitor core forms two parallel hydrogen bonds to the hinge region (CO of His107 and NH of Met109). To the best of our knowledge, this represents a new hinge region binding motif. In addition, the two NH's of the urea moiety are pointing toward the backbone CO of Met109 and serve as hydrogen-bonding donors. The aliphatic linker of the solubilizing pyrrolidine−butane moiety is surprisingly not pointing toward the solvent but rather folded inward toward the ATP pocket. The glycine-rich loop is relatively mobile in this complex and is partially not observed in the crystal structure. Therefore, the movement of the activation loop to its inactive conformation and its stabilization by **8** induces an upward movement of the acrylodan-labeled glycine-rich loop of p38α (see Figure 1) due to the cross-talk that exists between these two structural motifs. This explains the sensitive detection of **8** with this new FLiK approach as well as with our original FLiK approach in which the activation loop of p38α was labeled (data not shown).

In the p38α−**6** complex (Figure 3c), the quinazoline-based inhibitor surprisingly adopts a binding mode analogous to that of trisubstituted imidazoles such as **5** (SB203580).[14,35] The kinase is stabilized in the DFG-out conformation with its quinazoline core stacked between the glycine-rich loop and the DFG motif, thus explaining the detection of this compound using our new FLiK approach. However, unlike **5**, compound **6** does not contact the hinge region, and the attached acrylamide extends further in the direction of the position used to label the activation loop in our original FLiK approach.[14] Therefore, activation loop labeling at this position may be incompatible with the binding mode of **6** described here and might explain why we were not able to previously detect and report the binding of **6** to p38α

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using our original approach of labeling the activation loop. This unique binding mode differs significantly from those of previously reported structural analogues of **6** in complex with GSK3β[32] and calmodulin-dependent protein kinase 1D (PDB code: 2JC6), where the amino-pyrazole moiety of the inhibitor strongly interacts with the hinge region via three hydrogen bonds and the kinase is found in the DFG-in conformation. Our discovery of a completely new binding mode for quinazoline-based compounds together with the sensitive detection of the DFG-in binder **7** (described above) highlights the additional advantage of our new FLiK approach to detect binders that induce conformational changes in the glycine-rich loop. These types of compounds may stimulate the development of new generations of kinase inhibitors, particularly in kinases that do not readily adopt the DFG-out conformation.

4. Discussion

Kinase inhibitor drug discovery has progressed significantly in the past decade and resulted in almost a dozen marketed drugs.[5,8] However, several challenges such as drug resistance and the poor selectivity of classical ATP-competitive inhibitors remain prominent issues in the development of new kinase inhibitors. Since most of the chemical scaffolds that qualify as ATP-competitive ligands have already been discovered, classical kinase activity assays, which require the kinase to be phosphorylated and in the active conformation, are becoming obsolete for the discovery of novel hits that serve as lead structures for future medicinal chemistry and chemical biology research.[2] Active kinases are less likely to adopt inactive conformations, thereby lowering the affinities of DFG-out binders[22,36] and leading to an enrichment of Type I scaffold hits that bind to the DFG-in conformation. Current medicinal chemistry research is focused on identifying and developing inhibitors that target alternative binding sites and/or stabilize inactive kinase conformations,[2,37−40] highlighting the need for innovative new approaches to detect and characterize such ligands.

We previously reported the development of the FLiK assay as a method for detecting and discriminating Type II and Type III DFG-out binders by directly monitoring changes in the conformation of the activation loop (labeled with acrylodan) induced by the binding of such ligands. However, not all kinases can adopt the DFG-out conformation, and there is currently no method available to predict whether kinases of interest can even adopt the DFG-out conformation (aside from trial and error). The identification of such kinases will only be revealed over time as the activation loop FLiK approach is applied to more and more kinases. To circumvent some of these issues, we developed an alternative labeling strategy that may make FLiK applicable to a larger number of kinases.

Using the serine−threonine kinase p38α as a model system, we generated a second generation FLiK assay that takes advantage of the cross-talk between the activation loop and the glycine-rich loop of the kinase domain, allowing the detection and characterization of the time-dependent binding of Type II and Type III inhibitors. The binding of these ligands causes the activation loop to change conformation, which in turn alters the acrylodan-labeled glycine-rich loop conformation. For BIRB-796 (**1**), measured K_d, k_{on}, and k_{off} values obtained with this new approach were similar to published values determined by other methods[7] and were also comparable to those obtained by labeling the activation loop,[14] clearly suggesting that labeling kinases at the glycine-rich loop is a reliable alternative screening tool for detecting DFG-out-binding Type II and Type III ligands in kinases.

Although the FLiK approach was originally intended to identify Type II and Type III inhibitors that physically occupy the allosteric site available in the DFG-out conformation, we have also been able to sensitively detect several Type I ligands that contact the hinge region and stabilize the DFG-out conformation of p38α by labeling the glycine-rich loop with acrylodan. Details provided by protein X-ray crystallography (compound **8**) revealed that these Type I ligands stabilize the DFG-out conformation of p38α by occupying the hydrophobic subpocket beyond the gatekeeper residue as well as by forming hydrophobic interactions with residues of the DFG motif (Phe169) or at the N-terminal end of the activation loop, thereby leaving the allosteric site completely exposed. The identification of such Type I ligands should not be underestimated since they might qualify as starting points for further development into Type II inhibitors that extend in the direction of the less-conserved allosteric site.[6] Such a strategy was successfully applied to the tyrosine kinase cSrc previously. However, in that case, Type II inhibitors were developed from Type III hits and extended in the direction of the hinge region of the kinase.[25]

The key to being able to detect Type I DFG-out binders using FLiK approaches is the ability to perform screens using the unphosphorylated form of the kinase in the absence of both substrate and ATP. As mentioned above, the unphosphorylated form of the kinase is more likely to adopt the DFG-out conformation in which residues in the DFG motif or N-terminal regions of the activation loop can interact with the ligand and thus enhance affinity. This is in contrast to classical activity-based assays that require the phosphorylated kinase, which is more likely to be found in the DFG-in conformation, thereby lowering the affinities of DFG-out binders and making it less likely that such preferred hits are detected.[22,36] The established use of traditional activity-based assays in screening campaigns desensitizes the detection of DFG-out binders and could explain the lack of information in the literature about the binding of **8** to active (i.e., phosphorylated) kinases other than VEGFR2. The reported high specificity of **8** has led to its application as a VEGFR2 inhibitor in clinical trials to stop tumor growth and proliferation by inhibiting angiogenesis. Given the submicromolar affinities of **8** detected using unphosphorylated p38α with the FLiK approach, these findings could also stimulate further studies of this clinically relevant compound or close derivatives for the treatment of other kinase-associated diseases. Additionally, the structural information provided here for **8** in complex with p38α (i.e., new type of hinge contact) could stimulate further medicinal chemistry efforts to build on the affine portions of this molecule to extend into the adjacent allosteric site and generate more pharmacologically desirable Type II inhibitors that bind to inactive kinase conformations.

By labeling the glycine-rich loop, we not only achieve the goal of identifying DFG-out binders in applicable kinases but

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also allow the detection of Type I ligands that gain affinity for the DFG-in conformation by directly inducing conformational changes in the glycine-rich loop. This feature is the most important advantage of this new approach over our initial FLiK assay, in which the activation loop was labeled. By using glycine-rich labeled p38α, we sensitively detected Type I inhibitors such as **7**, which bind to the DFG-in conformation of p38α. Such compounds may gain affinity for the kinase by inducing changes in the conformation of the glycine-rich loop that help shield the ligand from the surrounding solvent.[19,20,41] Since the position in the glycine-rich loop responsible for these interactions is often conserved as an aromatic Tyr or Phe in more than 80% of kinases, this new labeling strategy extends the use of FLiK to additional kinases, including many kinases that are not regulated by a readily inducible DFG-in/out equilibrium. Detection of Type I inhibitors that interact with the glycine-rich loop may provide insights for the development of new scaffolds that take advantage of these interactions while avoiding the more traditional focus on identifying new types of hinge region contacts. Changes in glycine-rich loop conformation may also provide additional ways of improving Type I inhibitor specificities. Of course, the selection of which FLiK labeling strategy to employ will depend on the types of ligands that are ultimately desired for a particular kinase of interest and which labeling strategy the target kinase tolerates best.

5. Conclusion

We have developed a powerful alternative screening method for the rapid identification of inhibitors that (i) stabilize inactive kinase conformations and/or (ii) interact with the glycine-rich loop of the kinase domain. Using protein X-ray crystallography to validate screening results obtained with this new approach, we found that this assay detects not only Type I ligands that stabilize the DFG-out conformation by way of a unique binding mode but also ligands that directly interact with the glycine-rich loop, resulting in high-affinity binding. In comparison to our recently reported assay in which we labeled the activation loop,[11,14] this assay system also utilizes the unphosphorylated form of the kinase and provides a powerful alternative screening tool for sensitively detecting changes in kinase conformation that are correlated with the binding of Type II/III inhibitors. This approach also avoids manipulation of the activation loop, which may not be tolerated in some kinases. Moreover, it is not known whether most kinases can even adopt the DFG-out conformation, thereby making the binding assay presented here an attractive alternative approach for detecting and designing high-affinity Type I compounds that interact directly with the glycine-rich loop.

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Supporting Information Available: Experimental details for the synthesis of **6**; data collection and refinement statistics for p38α co-crystallized with **6**, **7**, and **8**; ATP K_m and IC_{50} values for different variants of p38α kinase; additional figures; and complete refs 15, 28, 30, 31, and 33. This material is available free of charge via the Internet at http://pubs.acs.org.

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