ACS chemical biology

Articles

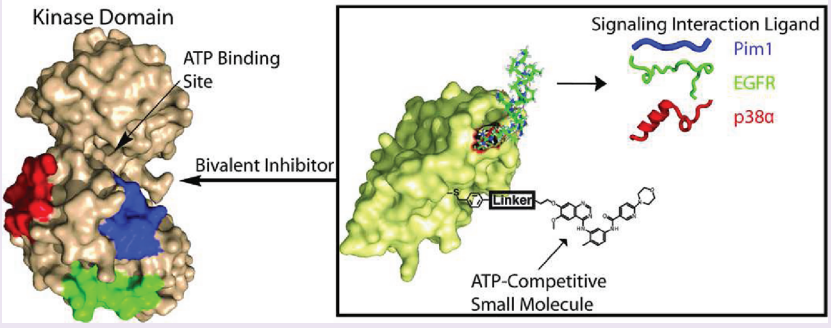
pubs.acs.org/acschemicalbiology

Targeting Diverse Signaling Interaction Sites Allows the Rapid Generation of Bivalent Kinase Inhibitors

Zachary B. Hill, B. Gayani K. Perera, Simeon S. Andrews, and Dustin J. Maly*

Department of Chemistry, University of Washington, Box 351700, Seattle, Washington 98195-1700, United States

Supporting Information



ABSTRACT: The identification of potent and selective modulators of protein kinase function remains a challenge, and new strategies are needed for generating these useful ligands. Here, we describe the generation of bivalent inhibitors of three unrelated protein kinases: the CAMK family kinase Pim1, the mitogen-activated protein kinase (MAPK) p38α, and the receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR). These bivalent inhibitors consist of an ATP-competitive inhibitor that is covalently tethered to an engineered form of the self-labeling protein O^6-alkylguanine-DNA alkyltransferase (SNAP-tag). In each example, SNAP-tag is fused to a peptide ligand that binds to a signaling interaction site of the kinase being targeted. These interactions increase the overall selectivity and potency of the bivalent inhibitors that were generated. The ability to exploit disparate binding sites in diverse kinases points to the generality of the method described. Finally, we demonstrate that ATP-competitive inhibitors that are conjugated to the bio-orthogonal tag O^4-benzyl-2-chloro-6-aminopyrimidine (CLP) are cell-permeable. The selective labeling of SNAP-tag with CLP conjugates allows the rapid assembly of bivalent inhibitors in living cells.

Protein phosphorylation cascades are a key component of most signaling events in eukaryotes.[1] Protein kinases, an enzyme family consisting of greater than 500 members, control intracellular protein phosphorylation by transferring the γ-phosphate of ATP to serine, threonine, or tyrosine residues of protein substrates. Because of the importance that these enzymes play in highly regulated cellular processes, aberrant kinase activity has been implicated in a number of diseases including diabetes, chronic inflammation, and cancer.[2,3] Reagents that allow the selective modulation of a single kinase in a cellular context are valuable tools for validating therapeutic targets and dissecting complex signaling pathways. However, because most potent inhibitors of kinase function interact with the highly conserved ATP-binding site, generating selective reagents remains a challenge. For this reason, there has been a great deal of interest in targeting interaction sites outside of the ATP-binding cleft.[4,5]

Highly selective bivalent inhibitors, which target at least one site outside of the ATP-binding cleft, have been identified for a number of kinases.[6−17] The most common class of bivalent inhibitors contain ligands that target both the ATP- and protein substrate-binding sites (bisubstrate inhibitors).[6−10] Potent bisubstrate inhibitors of protein kinase A (PKA), insulin receptor kinase (IRK), and AKT have been developed. In addition, bivalent inhibitors that interact with at least one site outside of the kinase active site have been described.[11−15] This strategy has been successful for identifying bivalent inhibitors of PKA, JNK, and the SRC-family kinases. In general, these inhibitors show increased potency and selectivity compared to that of the monovalent components that they are derived from. Recently, we have reported bivalent inhibitors of the highly homologous tyrosine kinases SRC and ABL.[16,17] These bivalent inhibitors contain an ATP-competitive small molecule inhibitor and a peptide ligand that targets the SRC Homology 3 (SH3) domains of these kinases. In contrast to previously described bivalent inhibitors, both ligands are displayed from a protein scaffold (Figure 1A). In order to generate these bivalent inhibitors, we have utilized an engineered form of the protein O^6-alkylguanine-DNA alkyltransferase (SNAP-tag), which is a self-labeling DNA repair enzyme. Johnsson and co-workers have generated SNAP-tag mutants that can be selectively and rapidly labeled with O^6-benzylguanine (BG) or O^4-benzyl-2-chloro-6-aminopyrimidine (CLP) derivatives in complex protein mixtures and in living cells.[18−25] BG- and CLP-

Received: September 22, 2011
Accepted: December 8, 2011
Published: December 8, 2011

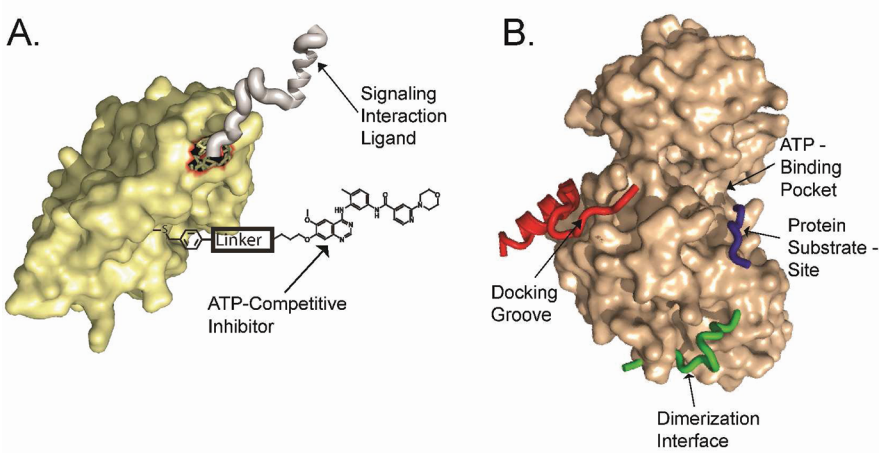


Figure 1. Bivalent SNAP-tag–small molecule conjugate inhibitors. (A) Schematic representation of bivalent inhibitors based on the SNAP-tag protein scaffold. (B) The general architecture of kinase catalytic domains with the ligands used in this study superimposed on this structure. The pseudosubstrate peptide used to target Pim1 is shown blue. The MIG6 peptide that binds to EGFR is shown in green. The 31-amino-acid peptide from MAPKAPK2 that binds to p38α is shown in red.

fluorophore conjugates are commercially available, and SNAP-tag labeling fusions have been used to study numerous aspects of protein function, including localization, trafficking, and turnover.[20−25] By conjugating a BG-linked ATP-competitive inhibitor to a genetically encoded SNAP-tag fusion that contains an SH3 domain ligand, we were able to rapidly generate potent and selective inhibitors of SRC and ABL.

A key aspect of this methodology is that selectivity is achieved through the interaction of the peptide ligand with a binding domain outside of the ATP-binding pocket. While it was possible to generate bivalent inhibitors that are able to discriminate between SRC and ABL by utilizing selective SH3 domain ligands, most kinases do not contain SH3 domains and cannot be targeted with bivalent inhibitors that utilize this signaling interaction. The highly conserved nature of the ATP-binding sites of protein kinases means that a suitable small molecule inhibitor can most likely be identified for any member of the kinome. However, for this methodology to be of general utility it is necessary that a wide range of ligands can be displayed from the SNAP-tag scaffold and be able to access a diverse range of kinase interaction sites. Here, we explore the general utility of SNAP-tag as a protein scaffold for generating bivalent kinase inhibitors. We demonstrate that multiple signaling interaction sites outside of the ATP-binding clefts of protein kinases can be effectively targeted and that these interactions allow high potency and selectivity to be achieved. Furthermore, we show that ATP-competitive inhibitors that are conjugated to CLP are cell-permeable and able to efficiently label SNAP-tag that is expressed in mammalian cells. The chemoselective reaction between SNAP-tag and CLP derivatives allows bivalent inhibitors to be assembled in living cells.

RESULTS AND DISCUSSION

To test the generality of the SNAP-tag scaffold for generating selective bivalent inhibitors, we selected three unrelated kinases: the CAMK family kinase Pim1, the mitogen-activated protein kinase (MAPK) p38α, and the receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR) kinase. Importantly, these kinases use different interactions to gain specificity in signaling. In addition, the spatial orientation of these interaction sites within the kinase domains of these enzymes vary. For each kinase, a genetically encoded ligand that targets a site outside of the ATP-binding pocket was selected for generating an SNAP-tag fusion protein (Figure 1B).

Bivalent Inhibitors of Pim1. Pim1 is a single domain serine/threonine kinase that is part of the Ca^{2+}/calmodulin-dependent protein kinase-related (CAMK) group. There are two additional members of the Pim kinase family, Pim2 and Pim3, which share a high degree of sequence homology with Pim1. Pim overexpression has been observed in a number of cancers, and these kinases are believed to contribute to some solid tumors and leukemias.[26] An interesting biochemical property of the Pim kinases is that they possess a much higher affinity for their protein and peptide substrates than most kinases.[27] Furthermore, the residues directly adjacent to the phospho-acceptor site are believed to be the primary determinants of Pim kinase substrate specificity. Therefore, we designed bisubstrate inhibitors that target the ATP- and protein substrate-binding sites of Pim1. To target the protein substrate site of Pim1, a peptide ligand that corresponds to a consensus substrate motif (Pimtide, ARKRRRHPSGPPTA) was selected.[27,28] This motif was converted into a pseudosubstrate by replacing both potential phosphorylation sites, serine and threonine, with alanine. Constructs that contain this pseudosubstrate linked to either the N- or C-terminus of SNAP-tag through a flexible serine-glycine linker were generated (SNAP(SIL1)–SNAP(SIL4), Figure 2A). Two SNAP-tag constructs, SNAP(SIL1) and SNAP(SIL3), were tested for their ability to inhibit Pim1 in an *in vitro* activity assay. Both SNAP-tag fusions have IC_{50}'s in the low micromolar range, which is consistent with the pseudosubstrate motifs of these constructs occupying the phospho-acceptor site of Pim1. The lack of any inhibition by wild type SNAP-tag demonstrates that the observed activity of these constructs is due to the pseudosubstrate peptide.

Having generated SNAP-tag fusion proteins that target Pim1, we next focused on developing an ATP-competitive inhibitor that is linked to a moiety, CLP, which is able to chemoselectively label the active site of SNAP-tag.[29] For this purpose, a derivative of the imidazo[1,2-*b*]pyridazine inhibitor SGI-1776 was generated (**1**, Figure 2B).[30] Based on a crystal structure of an analogue of SGI-1776 bound to Pim1, the piperidine group of this inhibitor was selected for linker attachment (Figure 2C).[31] CLP was tethered to SGI-1776 through a flexible

dx.doi.org/10.1021/cb200387g | *ACS Chem. Biol.* 2012, 7, 487–495

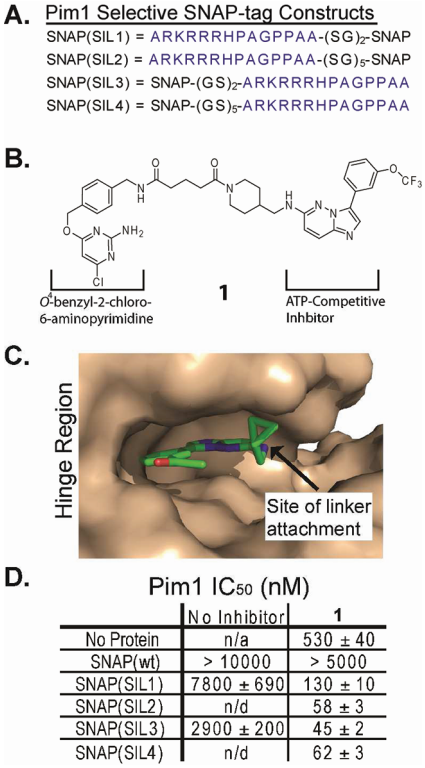


Figure 2. Bivalent inhibitors of Pim1. (A) SNAP-tag fusions that target Pim1 (SNAP(SIL1)-SNAP(SIL4)). The Pim1 pseudosubstrate is shown in blue. (B) CLP derivative **1**. (C) A crystal structure of an imidazo[1,2-*b*]pyridazine inhibitor bound to Pim1. (PDB code 2C3I) (D) *In vitro* activities of SNAP-tag fusions, CLP derivatives, and assembled SNAP-tag−small molecule conjugates against Pim1. All protein−small molecule conjugates were prepared in two independent labeling reactions. Values shown are the average of assays performed in quadruplicate ± SEM.

glutaric acid linker (Figure 2B). CLP-derivative **1** was then tested for its ability to inhibit the catalytic activity of Pim1 (Figure 2D). Although the IC_{50} of **1** for Pim1 (IC_{50} = 530 ± 40 nM) is higher than the value reported for SGI-1776 (IC_{50} = 7 nM), its potency is sufficient for use as a component of a bivalent inhibitor.

Next, the IC_{50}'s of assembled small molecule−SNAP-tag conjugates against Pim1 activity were determined (Figure 2D). Conjugation of inhibitor **1** to SNAP(wt) (Figure 2D, (SNAP(wt)-**1**)), led to a greater than 9-fold loss in overall potency (IC_{50} > 5000 nM) compared to CLP derivative **1**. However, conjugation of **1** to SNAP-tag constructs that contain a pseudosubstrate peptide leads to bivalent Pim1 inhibitors with much lower IC_{50} values (Figure 2D). SNAP(SIL3)-**1**, which contains the pseudosubstrate peptide at the C-terminus of SNAP-tag, is the most potent inhibitor, with an IC_{50} of 45 ± 2 nM against Pim1. This bivalent inhibitor is 12- and 60-fold more potent than the monovalent inhibitors **1** and SNAP-(SIL3), respectively. Most strikingly, inhibitor **1** has a >100-fold lower IC_{50} when displayed from an SNAP-tag construct that contains a pseudosubstrate peptide, SNAP(SIL3), compared with that of a SNAP-tag construct that does not, SNAP(wt). This demonstrates that bivalent inhibitors based on the SNAP-tag scaffold are able to effectively target the ATP- and protein substrate-binding sites of a kinase despite their close proximity.

Bivalent Inhibitors of p38α. p38α is a MAPK that is involved in a number of cellular stress response pathways. Like Pim1, p38α contains only a catalytic domain, which possesses all of the recognition elements necessary for substrate selection. To target the MAPK p38α, we focused on using ligands that bind to the docking groove. Activators and substrates of p38α bind tightly to this site, and docking domain interactions contribute to the high degree of signaling specificity of this kinase.[32−35] One such substrate is the mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2).[36−39] MAPKAPK2 binds tightly to p38α, and a 31-amino acid peptide derived from this protein occupies the docking groove of p38α without overlapping with the kinase active site.[37] Two SNAP-tag fusions that display the MAPKAPK2 peptide from their N-terminus were generated (SNAP(SIL5) and SNAP-(SIL6), Figure 3A) and tested for the ability to inhibit the

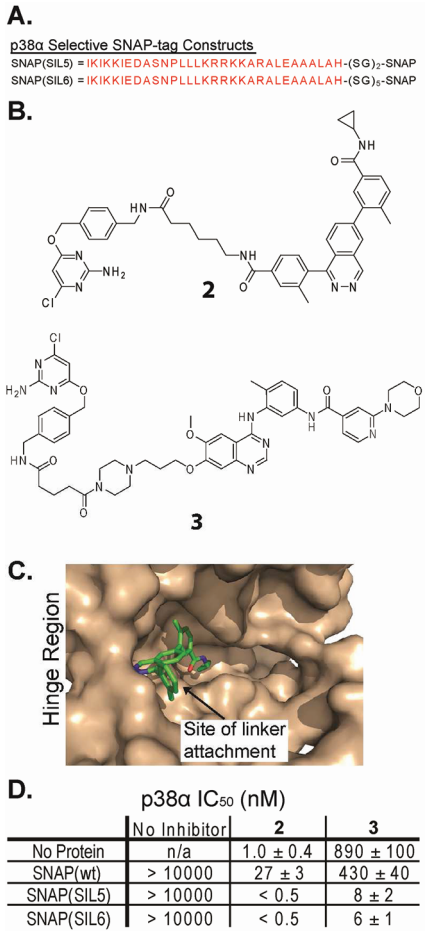


Figure 3. Bivalent inhibitors of p38α. (A) SNAP-tag fusions (SNAP(SIL5) and SNAP(SIL6)) that target p38α. The docking domain ligands that target p38α are shown in red. (B) CLP derivatives **2** and **3**. (C) The crystal structure of an inhibitor based on the phthalazine scaffold bound to p38α (PDB code 3DS6). (D) *In vitro* activities of SNAP-tag fusions, CLP derivatives, and assembled SNAP-tag−small molecule conjugates against p38α. All protein−small molecule conjugates were prepared in two independent labeling reactions. Values shown are the average of assays performed in quadruplicate ± SEM.

catalytic activity of p38α. As expected, neither SNAP-tag construct containing a docking domain groove ligand inhibits the activity of this kinase (Figure 3D).

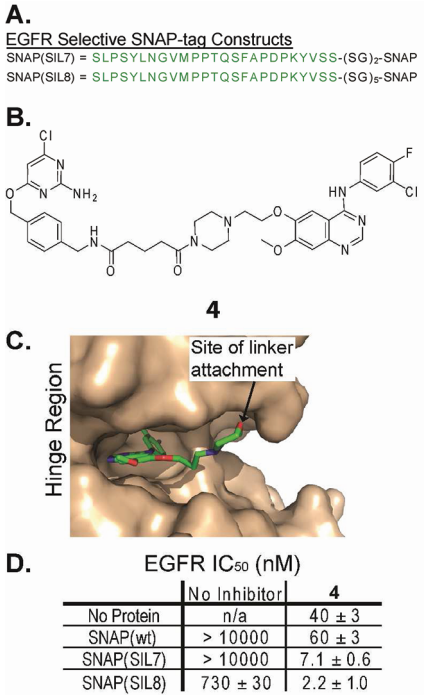
A number of selective ATP-competitive inhibitors that target p38α have been developed.[40−42] Therefore, we selected two

dx.doi.org/10.1021/cb200387g | *ACS Chem. Biol.* 2012, 7, 487−495

ATP-competitive inhibitors based on different scaffolds to develop CLP-conjugated inhibitors (Figure 3B). Derivative **2** contains a potent p38α inhibitor based on a phthalazine scaffold conjugated to CLP through a 6-aminohexanoic acid linker (Figure 3C).[41] The other derivative that targets p38α, **3**, is a 4-anilinoquinazoline inhibitor that is linked to CLP through a flexible tether attached to the C-7 position of this scaffold. The ATP-competitive inhibitor from conjugate **3** is unique from that of **2** in that it binds to an inactive conformation of p38α.[42,43] Both CLP derivatives **2** and **3** were tested for their ability to inhibit the catalytic activity of p38α in an *in vitro* activity assay (Figure 3D). Compound **2** is an extremely potent inhibitor of p38α and has an IC_{50} approaching the enzyme concentration used in the assay, whereas **3** has an IC_{50} in the high nanomolar range. The availability of two inhibitors with different affinities for the ATP-binding site of p38α provides the opportunity to tune the potency of assembled bivalent inhibitors.

The effect of conjugating inhibitors **2** and **3** to SNAP-tag was assessed in a p38α activity assay (Figure 3D). Consistent with the short tether length of derivative **2**, the potency of this inhibitor is significantly reduced (27-fold) when conjugated to SNAP(wt). In contrast, SNAP(wt)-**3** (IC_{50} = 430 ± 40 nM) is a slightly more potent inhibitor of p38α than unconjugated derivative **3** (IC_{50} = 890 ± 100 nM). Conjugating CLP derivative **2** to either SNAP-tag construct that contains a docking domain ligand (SNAP(SIL5) or SNAP(SIL6)) leads to at least a 50-fold increase in overall potency compared to that of SNAP(wt)-**2**. Unfortunately, the actual IC_{50}'s of SNAP-(SIL5)-**2** (IC_{50} < 0.5 nM) and SNAP(SIL6)-**2** (IC_{50} < 0.5 nM) could not be determined because the observed values approached the concentration of enzyme used in the activity assay. However, a more quantitative analysis of the contribution of docking domain ligand binding to bivalent inhibitor potency could be performed for bivalent inhibitors conjugated to CLP derivative **3**. SNAP(SIL5)-**3** and SNAP(SIL6)-**3** are >50-fold more potent against p38α activity than SNAP(wt)-**3**, consistent with these bivalent inhibitors occupying both the docking groove and ATP-binding site of this kinase. In addition, SNAP(SIL6)-**3** has a >100-fold lower IC_{50} compared with that of unconjugated CLP derivative **3**. These results demonstrate that a significant energetic contribution to bivalent inhibitor binding can be obtained by engaging the docking grooves of MAPKs. As selective docking domain ligands have been identified for a majority of the MAPKs, it should be possible to generate bivalent inhibitors based on the SNAP-tag scaffold for any member of this important class of kinases.[44]

Bivalent Inhibitors of EGFR. EGFR is a clinically important RTK that is activated by extracellular growth factors.[45] EGFR contains multiple domains, including an extracellular receptor domain, a transmembrane region, and a catalytic kinase domain. Upon growth factor binding, the kinase domain of EGFR is activated through dimerization, which results in phosphorylation of specific tyrosine residues in the C-terminal tail and recruitment of downstream effectors.[46] Unike p38α and Pim1, EGFR does not contain any high affinity interaction sites for its substrates. However, the C-lobe of this kinase possesses a hydrophobic dimer interface. To target this site on EGFR, we generated SNAP-tag fusions that contain a 25-amino-acid peptide from the mitogen-induced gene 6 (MIG6) protein (Figure 4A).[47] MIG6 blocks EGFR activation by inhibiting catalytic domain dimerization, and the 25-amino-acid peptide used in the SNAP-tag fusions binds to the



	No Inhibitor	4
No Protein	n/a	40 ± 3
SNAP(wt)	> 10000	60 ± 3
SNAP(SIL7)	> 10000	7.1 ± 0.6
SNAP(SIL8)	730 ± 30	2.2 ± 1.0

Figure 4. Bivalent inhibitors of EGFR. (A) SNAP-tag fusions that target EGFR (SNAP(SIL7) and SNAP(SIL8)). The 25-amino-acid MIG6 peptides are shown in green. (B) CLP derivative **4**. (C) The crystal structure of gefitinib bound to EGFR. (PDB code 2ITY). (D) *In vitro* activities of SNAP-tag fusions, CLP derivatives, and assembled SNAP-tag–small molecule conjugates against EGFR. All protein–small molecule conjugates were prepared in two independent labeling reactions. Values shown are the average of assays performed in quadruplicate ± SEM.

dimerization interface without occupying the active site of this kinase. SNAP-tag(wt) and both SNAP-tag fusions displaying the MIG6 peptide from their N-terminus (SNAP(SIL7) and SNAP(SIL8)) were tested for their ability to inhibit the catalytic activity of EGFR in an activity assay (Figure 4D). Although SNAP(wt) and SNAP(SIL7) do not inhibit EGFR at the highest concentration of protein tested (10 μM), SNAP(SIL8) is a weak inhibitor (IC_{50} = 730 ± 30 nM) of this kinase. The mechanism by which SNAP(SIL8) inhibits EGFR is unclear and currently under investigation. However, this result provides evidence that at least one of the SNAP-tag fusions is able to interact with EGFR.

To target the ATP-binding site of EGFR, we generated an analogue of the clinically approved drug gefitinib.[48] Gefitinib is a potent and selective EGFR inhibitor based on the 4-anilinoquinazoline scaffold. A CLP-derivatized version of gefitinib was generated by modifying the 6-position of the 4-anilinoquinazoline scaffold with a linker (Figure 4B and C). CLP derivative **4** has a similar IC_{50} for EGFR as gefitinib, demonstrating that attaching a linker to this position does not appear to affect interaction with the catalytic domain of this kinase (Figure 4D).

The ability of assembled SNAP-tag–small molecule conjugates to inhibit EGFR was next tested (Figure 4D). Similar to 4-anilinoquinazoline **3**, the IC_{50} of **4** is minimally affected when it is conjugated to SNAP(wt) (SNAP(wt)-**4**, IC_{50} = 60 ± 3 nM). However, conjugation of **4** to either of the MIG6 peptide-containing SNAP-tag constructs results in significantly more potent bivalent inhibitors. The most potent bivalent inhibitor,

dx.doi.org/10.1021/cb200387g | *ACS Chem. Biol.* 2012, 7, 487–495

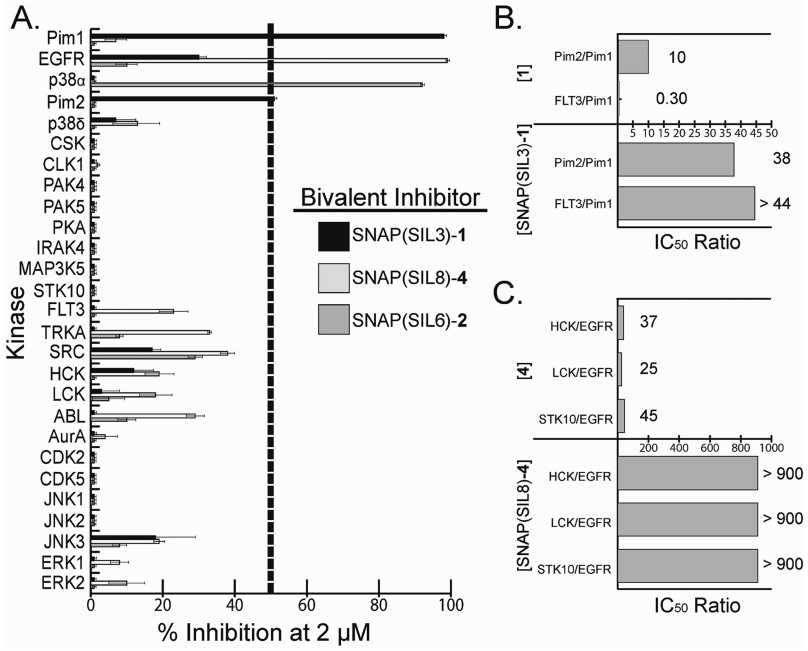


Figure 5. Activity of SNAP(SIL3)-**1**, SNAP(SIL6)-**2**, and SNAP(SIL8)-**4** against a panel of 27 protein kinases. (A) Percent inhibition was measured at a concentration of 2 μM for each construct. Values shown are the average of assays performed in triplicate ± SEM (B) IC_{50} ratios of **1** and SNAP(SIL3)-**1** against Pim1, Pim2, and FLT3. (C) IC_{50} ratios of **4** and SNAP(SIL8)-**4** against EGFR, HCK, LCK, and STK10.

SNAP(SIL8)-**4**, has an IC_{50} 28-fold lower than that of monovalent SNAP-tag construct SNAP(wt)-**4** and 18-fold lower than that of CLP derivative **4** (Figure 4D). Hence, the interaction of the MIG6 peptide displayed from SNAP-tag with EGFR clearly increases the potencies of both bivalent inhibitors.

Selectivity of Bivalent Inhibitors. After identifying potent bivalent inhibitors of Pim1, p38α, and EGFR, we next determined the kinase selectivity of these protein–small molecule conjugates (Figure 5A and Supplementary Figure S1). To do this, the most potent bivalent inhibitors of Pim1, p38α, and EGFR (SNAP(SIL3)-**1**, SNAP(SIL6)-**2**, and SNAP-(SIL8)-**4**) were tested against a panel of 27 kinases. Kinases in this screen were selected for diversity and on the basis of the likelihood that they are off-targets of the ATP-competitive inhibitors **1**, **2**, and **4**. The MAP kinases p38δ, JNK1, JNK2, JNK3, ERK1, and ERK2 were used as counter-screens for the p38α inhibitor **2** because they possess highly homologous active sites. Pim2 and the receptor tyrosine kinase FLT3 were included in the panel because these kinases have previously been demonstrated to be inhibited by SGI-1776 (the inhibitor that CLP analogue **1** is derived from).[30] The STE20 kinase STK10 and the nonreceptor tyrosine kinases LCK and HCK were selected because these kinases were identified in a large-scale selectivity study as off-targets of the drug gefitinib.[49] The results of screening all three bivalent inhibitors at a single concentration (2 μM) against the selectivity panel are shown in Figure 5A. Both SNAP(SIL6)-**2** and SNAP(SIL8)-**4** are very selective for their respective targets p38α and EGFR, demonstrating an IC_{50} of >2 μM for all of the other kinases in the panel. The only kinase that the bivalent Pim1 inhibitor SNAP(SIL3)-**1** shows significant activity against is Pim2, but at a reduced potency (IC_{50}(Pim1) = 45 ± 2; IC_{50}(Pim2) = 1700 ± 300). Thus, all three potent bivalent inhibitors demonstrate a high degree of selectivity for their respective targets.

To further understand the contribution of signaling site interactions toward bivalent inhibitor selectivity, we performed a more quantitative activity analysis against a focused panel of kinases (Figure 5B and C). To do this, the ratio of the activity of each monovalent CLP derivative for its desired target *versus* an off-target was determined and compared to the ratio for the corresponding assembled bivalent inhibitor. CLP derivative **1** is a slightly more potent inhibitor of FLT3 than Pim1 ([IC_{50}(FLT3)/IC_{50}(Pim1)] = 0.3) but 10-fold selective for Pim1 over Pim2 ([IC_{50}(Pim2)/IC_{50}(Pim1)] = 10). Conjugating inhibitor **1** to an SNAP-tag construct containing a pseudosubstrate peptide motif increases the overall selectivity ratio for both targets. SNAP(SIL3)-**1** is >44-fold more potent against Pim1 (IC_{50} = 45 ± 2 nM) than FLT3 (IC_{50} = > 2000 nM), and thus the FLT3/Pim1 selectivity ratio is >130-fold more favorable for the bivalent inhibitor than monovalent ATP-competitive inhibitor **1**. A similar but less pronounced trend is observed for Pim2; SNAP(SIL3)-**1** has a 4-fold more favorable Pim2/Pim1 selectivity ratio compared with that of **1**. In this construct, both the pseudosubstrate peptide and the ATP-competitive inhibitor contribute to the observed 38-fold overall selectivity of the bivalent inhibitor. Next, the ability of gefitinib analogue **4** to inhibit STK10, HCK, and LCK was determined. While monovalent CLP derivative **4** is an inhibitor of moderate potency against these kinases (HCK (IC_{50} = 1500 ± 100 nM), LCK (IC_{50} = 1000 ± 100 nM), and STK10 (IC_{50} = 1800 ± 100 nM)), it still demonstrates high selectivity for EGFR ([IC_{50}(STK10)/IC_{50}(EGFR)] = 45; [IC_{50}(LCK)/IC_{50}(EGFR)] = 25; ([IC_{50}(STK10)/IC_{50}(HCK)] = 37). However, conjugating inhibitor **4** to SNAP(SIL8) increases the observed selectivity for EGFR to >900-fold for all three kinases (bottom panel, Figure 5C). The increased potency of the bivalent inhibitor against EGFR due to the specific interaction with MIG6 contributes to the high degree of selectivity observed. Finally, monovalent p38α inhibitor **2** was found to be >1000-fold selective for its target over all of the

dx.doi.org/10.1021/cb200387g | *ACS Chem. Biol.* 2012, 7, 487–495

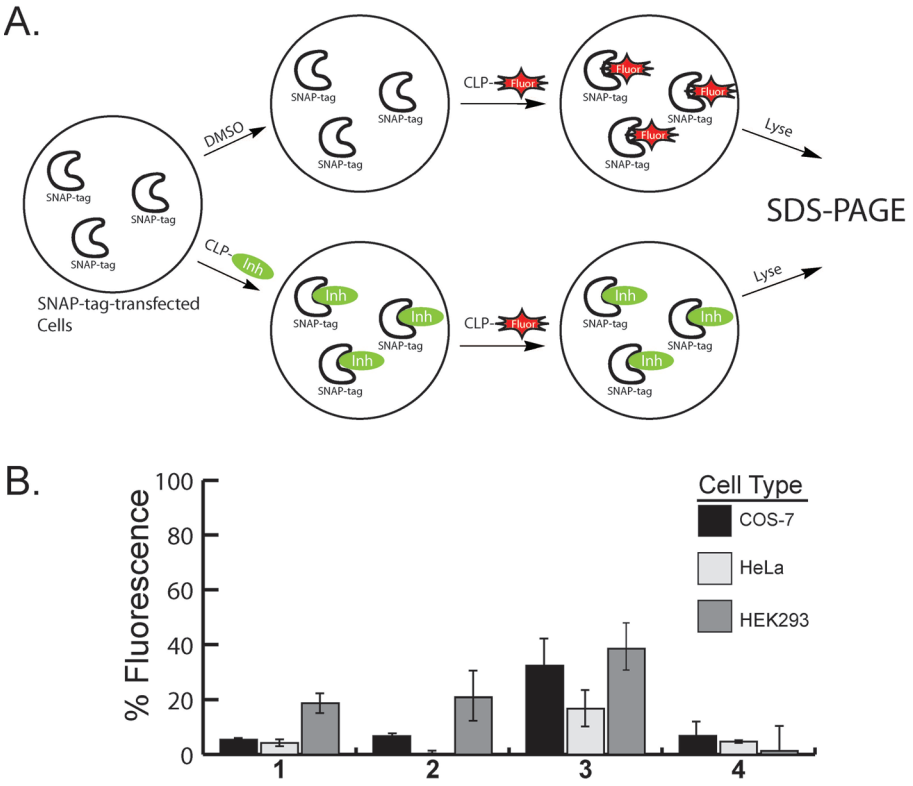


Figure 6. Labeling of SNAP-tag in cells by **1**, **2**, **3**, and **4**. (A) SNAP(wt) transfected cells were incubated with 10 μM **1**, **2**, **3**, **4**, or DMSO for 1 h, followed by incubation with CLP-rhodamine (3 μM) for 30 min. (B) Fluorescence relative to a DMSO control was measured and corrected for protein expression. Reduction in fluorescence, relative to DMSO, indicates successful labeling of SNAP-tag by **1**, **2**, **3**, and **4**. Values shown are the average of assays performed in duplicate ± SEM.

other kinases tested. Therefore, it was not possible to perform a similar analysis. However, the lack of inhibition observed for bivalent inhibitor SNAP(SIL6)-**2** against the MAPKs p38δ, JNK1, JNK2, JNK3, ERK1, and ERK2 demonstrates that targeting the docking domain of p38α does not compromise the inherent selectivity of the ATP-competitive inhibitor attached to the SNAP-tag scaffold.

Cell Permeability of CLP-Linked Inhibitors. For bivalent inhibitors based on the SNAP-tag scaffold to be of general utility for studying kinase function, they must be able to be assembled in living cells. While previous studies have shown that fluorophores that are conjugated to BG or CLP are able to specifically label SNAP-tag in mammalian cells, it is unclear how cell-permeable the ATP-competitive derivatives used in this study are. While it would be possible to develop radiolabeled analogues of each CLP derivative to determine intracellular labeling of SNAP-tag, we wished to develop a general method that can be used to measure the cell permeability of a number of compounds without the need for additional synthetic effort. Therefore, we developed a general, fluorescent intracellular blocking assay to determine the cell permeability of conjugates **1**–**4** (Figure 6A).

Mammalian cells that were transiently transfected with SNAP-tag were first incubated with derivatives **1**, **2**, **3**, or **4** for 1 h. After 1 h, any unmodified SNAP-tag active sites were then labeled with the cell-permeable fluorophore CLP-rhodamine. The extent of SNAP-tag labeling was then determined by lysing the cells, subjecting the lysate to SDS-PAGE, and quantifying the fluorescence intensity of SNAP-tag. A decrease in SNAP-tag fluorescence indicates that an analogue is cell-permeable and able to label intracellular SNAP-tag, thus blocking conjugation to CLP-rhodamine. The results of blocking studies with derivatives **1**–**4** for three commonly used cell lines (COS-7, HeLa, and HEK293) are shown in Figure 6B. Gratifyingly, all four conjugates block rhodamine labeling of SNAP-tag and appear to be cell-permeable. Only derivative **3**, which contains a very large ATP-competitive inhibitor, does not label >80% of the available SNAP-tag in all three cell lines. Furthermore, the ability of derivatives **1**–**4** to block intracellular labeling of SNAP-tag has also been verified by live cell imaging (data not shown).

After assembling bivalent SNAP-tag conjugates in cells, it will be necessary to remove any excess CLP derivative that is not conjugated to SNAP-tag. We did not envision this being a problem because previous studies have demonstrated that it is possible to efficiently washout unconjugated BG- or CLP-fluorophore derivatives from cells after labeling.[50] However, to guarantee that CLP-inhibitor derivatives can efficiently be removed from cells, washout experiments with CLP derivative **4** were performed (Supplementary Figure S2). Epidermal growth factor (EGF) stimulation of cells that have been transiently transfected with EGFR was used as a functional assay for kinase inhibition. Stimulation of serum-starved cells with EGF in the presence of inhibitor **4** (10 μM) led to a significant decrease in EGFR autophosphorylation (phospho-Y1068) compared to a DMSO control (Supplementary Figure S2). The level of EGFR autophosphorylation inhibition observed in the presence of **4** was similar to that of gefitinib (data not shown). In contrast, cells that were first incubated with inhibitor **4** (10 μM) and then subjected to washout conditions prior to EGF stimulation demonstrated EGFR autophosphorylation (phospho-Y1068) levels that are identical

dx.doi.org/10.1021/cb200387g | *ACS Chem. Biol.* 2012, 7, 487–495

to that of the DMSO control. This clearly demonstrates the CLP conjugate **4** can effectively be removed from cells using standard washout conditions. The ability to both assemble SNAP-tag-based bivalent inhibitors in cells and to wash out any noncovalently bound CLP conjugates greatly expands the utility of these reagents for studying kinase signaling pathways.

Conclusions. In summary, we have expanded the diversity of protein kinases that can be selectively targeted with bivalent inhibitors based on the SNAP-tag protein scaffold. All of the bivalent inhibitors that were generated in this study contain an ATP-competitive inhibitor that is conjugated to a SNAP-tag fusion protein. Peptide ligands displayed from the SNAP-tag protein scaffold are able to exploit disparate interaction sites outside of the ATP-binding clefts of EGFR, Pim1, and p38α. Importantly, the sites that these ligands target are the same that EGFR, Pim1, and p38α use to obtain specificity in cellular signaling events. The ability to use functionally divergent interaction sites should allow this strategy to be applied to any protein kinase for which a suitable ligand can be identified. In addition, we have demonstrated that CLP-inhibitor conjugates are cell-permeable. This allows for the rapid assembly of bivalent inhibitors in living cells. Furthermore, by using SNAP-tag fusions that contain localization sequences it will be possible to generate bivalent inhibitors in specific subcellular compartments. These localized inhibitors can be used to probe subcellular kinase function, which is not possible with traditional pharmacological agents or genetic techniques. The use of these reagents to study specific cellular signaling events is ongoing.

METHODS

Synthesis of Compounds 1, 2, 3, and 4. Synthetic schemes, detailed procedures and characterization of compounds **1**, **2**, **3**, and **4** can be found in the Supporting Information.

General Procedure for Generating SNAP-tag Fusion Proteins. Overlap extension PCR was performed as previously described.[16]

SNAP-tag Expression and Purification. SNAP-tag fusions were expressed and purified using a previously published procedure.[16] All proteins were analyzed with a Bruker Esquire Ion Trap Mass Spectrometer. Spectra can be found in the Supporting Information.

Preparation of SNAP-tag Fusion Small Molecule Conjugates. SNAP(wt) and all SNAP-tag fusions were labeled with **1**, **2**, **3**, or **4** and purified using a previously published procedure.[16]

General Activity Assay Conditions. Pim1, p38α, and EGFR were all assayed using general activity assay conditions previously described.[16]

Assay Condition for Selectivity Panel. Inhibitors (concentration =2 μM) were assayed in triplicate against Pim2, p38δ, CSK, CLK1, PAK4, PAK5, PKA, IRAK4, MAP3K5, STK10, FLT3, TRKA, SRC, HCK, LCK, ABL, AurA, CDK2, CDK5, JNK1, JNK2, JNK3, ERK1, and ERK2. General activity assay conditions were used. Details of each assay can be found in the Supporting Information.

General Cellular Assay Information. COS-7 cells were cultured in Gibco High Glucose DMEM Media supplemented with 10% fetal bovine serum and penicillin/streptomycin. HeLa cells were cultured in Gibco Low Glucose DMEM Media supplemented with 10% fetal bovine serum and penicillin/streptomycin. HEK-293 cells were cultured in Gibco RPMI-1640 Media supplemented with 10% fetal bovine serum and penicillin/streptomycin. All cells were incubated at 37 °C and 5% CO_2.

Blocking and Labeling. SNAP-tag transfected cells were incubated with 10 μM concentrations of **1**, **2**, **3**, **4**, CLP-alkyne (see structure in the Supporting Information), or 0.5% DMSO (control) in complete media for 1 h. The media was then aspirated and the cells were incubated with 3 μM of CLP-rhodamine (see structure in the Supporting Information) in complete media for 30 min. Cells were washed 3 times with complete media and then incubated in media for 30 min to remove any unbound CLP-rhodamine. Cells were lysed with 50 μL of 1X SDS loading buffer. Samples were run on a 15% SDS-PAGE gel and scanned with a GE Typhoon FLA 9000 scanner to measure fluorescence. SNAP-tag expression levels of samples were analyzed by immunoblotting with an anti-His6 antibody (ABM Inc.). Fluorescence levels were corrected for SNAP-tag expression levels. Fluorescence levels from cells pretreated with CLP-alkyne were considered to be the maximum blocking possible and so were subtracted from all other values. Percent fluorescence was calculated by dividing the corrected fluorescence level for inhibitor-treated cells by the corrected fluorescence levels of DMSO-treated cells. Reduced fluorescence relative to that of DMSO-treated cells indicates that all SNAP-tag active sites were successfully labeled by **1**, **2**, **3**, or **4** and, therefore, indicates cell permeability. All labeling was performed and analyzed in duplicate.

ASSOCIATED CONTENT

Supporting Information

Supplementary figures, experimental methods, and characterization of conjugates **1**−**4**. This material is available free of charge *via* the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: maly@chem.washington.edu.

ACKNOWLEDGMENTS

D.J.M. acknowledges support from the National Institutes of Health in the form of Grant R01GM086858. We thank M. Seeliger (SUNY, Stony Brook) for providing expression plasmids for ABL, HCK, LCK, and SRC.

REFERENCES

(1) Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science 298*, 1912−1934.

(2) Blume-Jensen, P., and Hunter, T. (2001) Oncogenic kinase signalling. *Nature 411*, 355−365.

(3) Cohen, P. (2002) Protein kinases—the major drug targets of the twenty-first century? *Nat. Rev. Drug Discovery 1*, 309−315.

(4) Parang, K., and Cole, P. A. (2002) Designing bisubstrate analog inhibitors for protein kinases. *Pharmacol. Ther. 93*, 145−157.

(5) Cox, K. J., Shomin, C. D., and Ghosh, I. (2011) Tinkering outside the kinase ATP box: allosteric (type IV) and bivalent (type V) inhibitors of protein kinases. *Future Med. Chem. 3*, 29−43.

(6) Ricouart, A., Gesquiere, J. C., Tartar, A., and Sergheraert, C. (1991) Design of potent protein kinase inhibitors using the bisubstrate approach. *J. Med. Chem. 34*, 73−78.

(7) Hines, A. C., and Cole, P. A. (2004) Design, synthesis, and characterization of an ATP-peptide conjugate inhibitor of protein kinase A. *Bioorg. Med. Chem. Lett. 14*, 2951−2954.

(8) Hines, A. C., Parang, K., Kohanski, R. A., Hubbard, S. R., and Cole, P. A. (2005) Bisubstrate analog probes for the insulin receptor protein tyrosine kinase: molecular yardsticks for analyzing catalytic mechanism and inhibitor design. *Bioorg. Chem. 33*, 285−297.

(9) Schneider, T. L., Mathew, R. S., Rice, K. P., Tamaki, K., Wood, J. L., and Schepartz, A. (2005) Increasing the kinase specificity of k252a by protein surface recognition. *Org. Lett. 7*, 1695−1698.

(10) Lee, J. H., Kumar, S., and Lawrence, D. S. (2008) Stepwise combinatorial evolution of Akt bisubstrate inhibitors. *ChemBioChem 9*, 507−509.

(11) Meyer, S. C., Shomin, C. D., Gaj, T., and Ghosh, I. (2007) Tethering small molecules to a phage display library: discovery of a selective bivalent inhibitor of protein kinase A. *J. Am. Chem. Soc. 129*, 13812−13813.

(12) Shomin, C. D., Meyer, S. C., and Ghosh, I. (2009) Staurosporine tethered peptide ligands that target cAMP-dependent protein kinase (PKA): optimization and selectivity profiling. *Bioorg. Med. Chem. 17*, 6196−6202.
(13) Stebbins, J. L., De, S. K., Pavlickova, P., Chen, V., Machleidt, T., Chen, L. H., Kuntzen, C., Kitada, S., Karin, M., and Pellecchia, M. (2011) Design and characterization of a potent and selective dual ATP- and substrate-competitive subnanomolar bidentate c-Jun N-terminal kinase (JNK) inhibitor. *J. Med. Chem. 54*, 6206−6214.
(14) Hah, J. M., Sharma, V., Li, H., and Lawrence, D. S. (2006) Acquisition of a "Group A"-selective Src kinase inhibitor via a global targeting strategy. *J. Am. Chem. Soc. 128*, 5996−5997.
(15) Profit, A. A., Lee, T. R., Niu, J., and Lawrence, D. S. (2001) Molecular rulers: an assessment of distance and spatial relationships of Src tyrosine kinase Sh2 and active site regions. *J. Biol. Chem. 276*, 9446−9451.
(16) Hill, Z. B., Perera, B. G., and Maly, D. J. (2009) A chemical genetic method for generating bivalent inhibitors of protein kinases. *J. Am. Chem. Soc. 131*, 6686−6688.
(17) Hill, Z. B., Perera, B. G., and Maly, D. J. (2011) Bivalent inhibitors of the tyrosine kinases ABL and SRC: determinants of potency and selectivity. *Mol. BioSyst. 7*, 447−456.
(18) Juillerat, A., Gronemeyer, T., Keppler, A., Gendreizig, S., Pick, H., Vogel, H., and Johnsson, K. (2003) Directed evolution of O^6-alkylguanine-DNA alkyltransferase for efficient labeling of fusion proteins with small molecules in vivo. *Chem. Biol. 10*, 313−317.
(19) Keppler, A., Gendreizig, S., Gronemeyer, T., Pick, H., Vogel, H., and Johnsson, K. (2003) A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nat. Biotechnol. 21*, 86−89.
(20) Keppler, A., Kindermann, M., Gendreizig, S., Pick, H., Vogel, H., and Johnsson, K. (2004) Labeling of fusion proteins of O^6-alkylguanine-DNA alkyltransferase with small molecules in vivo and in vitro. *Methods 32*, 437−444.
(21) Keppler, A., Pick, H., Arrivoli, C., Vogel, H., and Johnsson, K. (2004) Labeling of fusion proteins with synthetic fluorophores in live cells. *Proc. Natl. Acad. Sci. U.S.A. 101*, 9955−9959.
(22) Pick, H., Jankevics, H., and Vogel, H. (2007) Distribution plasticity of the human estrogen receptor alpha in live cells: distinct imaging of consecutively expressed receptors. *J. Mol. Biol. 374*, 1213−1223.
(23) Jansen, L. E., Black, B. E., Foltz, D. R., and Cleveland, D. W. (2007) Propagation of centromeric chromatin requires exit from mitosis. *J. Cell Biol. 176*, 795−805.
(24) McMurray, M. A., and Thorner, J. (2008) Septin stability and recycling during dynamic structural transitions in cell division and development. *Curr. Biol. 18*, 1203−1208.
(25) Bojkowska, K., Santoni de Sio, F., Barde, I., Offner, S., Verp, S., Heinis, C., Johnsson, K., and Trono, D. (2011) Measuring in vivo protein half-life. *Chem. Biol. 18*, 805−815.
(26) Shah, N., Pang, B., Yeoh, K. G., Thorn, S., Chen, C. S., Lilly, M. B., and Salto-Tellez, M. (2008) Potential roles for the PIM1 kinase in human cancer−a molecular and therapeutic appraisal. *Eur. J. Cancer 44*, 2144−2151.
(27) Bullock, A. N., Debreczeni, J., Amos, A. L., Knapp, S., and Turk, B. E. (2005) Structure and substrate specificity of the Pim-1 kinase. *J. Biol. Chem. 280*, 41675−41682.
(28) Hutti, J. E., Jarrell, E. T., Chang, J. D., Abbott, D. W., Storz, P., Toker, A., Cantley, L. C., and Turk, B. E. (2004) A rapid method for determining protein kinase phosphorylation specificity. *Nat. Methods 1*, 27−29.
(29) For this study, weused O^4-benzyl-2-chloro-6-aminopyrimidine(CLP), which is able to selectively label SNAP-tag, instead of O^6-benzylguanine (BG). We have found that ATP-competitiveinhibitors conjugated to CLP are much more cell-permeable than thosethat are linked to BG. The difference in cell permeability betweenthe CLP and BG tags has previously been reported: Srikun, D., Albers, A. E., Nam, C. I., Iavarone, A. T., and Chang, C. J. (2010) Organelle-targetable fluorescent probes for imaging hydrogen peroxide in living cells via SNAP-Tag protein labeling. *J. Am. Chem. Soc. 132*, 4455−4465.
(30) Chen, L. S., Redkar, S., Bearss, D., Wierda, W. G., and Gandhi, V. (2009) Pim kinase inhibitor, SGI-1776, induces apoptosis in chronic lymphocytic leukemia cells. *Blood 114*, 4150−4157.
(31) Pogacic, V., Bullock, A. N., Fedorov, O., Filippakopoulos, P., Gasser, C., Biondi, A., Meyer-Monard, S., Knapp, S., and Schwaller, J. (2007) Structural analysis identifies imidazo[1,2-*b*]pyridazines as PIM kinase inhibitors with in vitro antileukemic activity. *Cancer Res. 67*, 6916−6924.
(32) Tanoue, T., Maeda, R., Adachi, M., and Nishida, E. (2001) Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions. *EMBO J. 20*, 466−479.
(33) Chang, C. I., Xu, B. E., Akella, R., Cobb, M. H., and Goldsmith, E. J. (2002) Crystal structures of MAP kinase p38 complexed to the docking sites on its nuclear substrate MEF2A and activator MKK3b. *Mol. Cell 9*, 1241−1249.
(34) Holland, P. M., and Cooper, J. A. (1999) Protein modification: docking sites for kinases. *Curr. Biol. 9*, R329−331.
(35) Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat. Cell Biol. 2*, 110−116.
(36) Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell 78*, 1027−1037.
(37) Lukas, S. M., Kroe, R. R., Wildeson, J., Peet, G. W., Frego, L., Davidson, W., Ingraham, R. H., Pargellis, C. A., Labadia, M. E., and Werneburg, B. G. (2004) Catalysis and function of the p38 alpha.MK2a signaling complex. *Biochemistry 43*, 9950−9960.
(38) ter Haar, E., Prabhakar, P., Liu, X., and Lepre, C. (2007) Crystal structure of the p38 alpha-MAPKAP kinase 2 heterodimer. *J. Biol. Chem. 282*, 9733−9739.
(39) White, A., Pargellis, C. A., Studts, J. M., Werneburg, B. G., and Farmer, B. T. 2nd (2007) Molecular basis of MAPK-activated protein kinase 2:p38 assembly. *Proc. Natl. Acad. Sci. U.S.A. 104*, 6353−6358.
(40) Mayer, R. J., and Callahan, J. F. (2006) p38 MAP kinase inhibitors: A future therapy for inflammatory diseases. *Drug Discovery Today: Ther. Strategies 3*, 49−54.
(41) Herberich, B., Cao, G. Q., Chakrabarti, P. P., Falsey, J. R., Pettus, L., Rzasa, R. M., Reed, A. B., Reichelt, A., Sham, K., Thaman, M., Wurz, R. P., Xu, S., Zhang, D., Hsieh, F., Lee, M. R., Syed, R., Li, V., Grosfeld, D., Plant, M. H., Henkle, B., Sherman, L., Middleton, S., Wong, L. M., and Tasker, A. S. (2008) Discovery of highly selective and potent p38 inhibitors based on a phthalazine scaffold. *J. Med. Chem. 51*, 6271−6279.
(42) Cumming, J. G., McKenzie, C. L., Bowden, S. G., Campbell, D., Masters, D. J., Breed, J., and Jewsbury, P. J. (2004) Novel, potent and selective anilinoquinazoline and anilinopyrimidine inhibitors of p38 MAP kinase. *Bioorg. Med. Chem. Lett. 14*, 5389−5394.
(43) Sullivan, J. E., Holdgate, G. A., Campbell, D., Timms, D., Gerhardt, S., Breed, J., Breeze, A. L., Bermingham, A., Pauptit, R. A., Norman, R. A., Embrey, K. J., Read, J., VanScyoc, W. S., and Ward, W. H. (2005) Prevention of MKK6-dependent activation by binding to p38alpha MAP kinase. *Biochemistry 44*, 16475−16490.
(44) Bardwell, A. J., Frankson, E., and Bardwell, L. (2009) Selectivity of docking sites in MAPK kinases. *J. Biol. Chem. 284*, 13165−13173.
(45) Citri, A., and Yarden, Y. (2006) EGF-ERBB signalling: towards the systems level. *Nat. Rev. Mol. Cell Biol. 7*, 505−516.
(46) Zhang, X., Gureasko, J., Shen, K., Cole, P. A., and Kuriyan, J. (2006) An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell 125*, 1137−1149.
(47) Zhang, X., Pickin, K. A., Bose, R., Jura, N., Cole, P. A., and Kuriyan, J. (2007) Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface. *Nature 450*, 741−744.
(48) Muhsin, M., Graham, J., and Kirkpatrick, P. (2003) Gefitinib. *Nat. Rev. Drug Discovery 2*, 515−516.

(49) Karaman, M. W., Herrgard, S., Treiber, D. K., Gallant, P., Atteridge, C. E., Campbell, B. T., Chan, K. W., Ciceri, P., Davis, M. I., Edeen, P. T., Faraoni, R., Floyd, M., Hunt, J. P., Lockhart, D. J., Milanov, Z. V., Morrison, M. J., Pallares, G., Patel, H. K., Pritchard, S., Wodicka, L. M., and Zarrinkar, P. P. (2008) A quantitative analysis of kinase inhibitor selectivity. *Nat. Biotechnol. 26*, 127−132.

(50) Juillerat, A., Heinis, C., Sielaff, I., Barnikow, J., Jaccard, H., Kunz, B., Terskikh, A., and Johnsson, K. (2005) Engineering substrate specificity of O^6-alkylguanine-DNA alkyltransferase for specific protein labeling in living cells. *ChemBioChem 6*, 1263−1269.