Title
Site-Directed Ligand Discovery for Allosteric Sites and Protein-Protein Interfaces

Permalink
https://escholarship.org/uc/item/3w59v6wn

Author
Rettenmaier, Terry Justin

Publication Date
2015

Peer reviewed|Thesis/dissertation
Site-Directed Ligand Discovery
for Allosteric Sites and Protein-Protein Interfaces

by

Terry Justin Rettenmaier

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry and Chemical Biology

in the

GRADUATE DIVISION
Acknowledgements

First, I want to thank my research advisor Jim Wells for always giving me the freedom, support, and enthusiasm I needed to develop and explore my own research interests. I feel incredibly lucky to have been a part of the diverse and stimulating lab environment that Jim has crafted where new ideas and fresh perspectives always abound. Coming to lab never felt like work, because the Wellsome was my second family. I will cherish the memories of our annual ski trip, Giants game, Christmas party, Super Bowls, NBA Finals, and weekly poker nights.

I am thankful to the all the members of the Wells lab and the SMDC who have each influenced me and guided me at various points during my time here. In particular, I want to thank Jack Sadowsky, Julie Zorn, Nathan Thomsen, Zach Hill, and Olivier Julien. I am especially thankful to our lab administrator Marja Tarr who treats each of us like family and always makes time to listen to our problems and share her wisdom. I also thank my thesis committee: Kevan Shokat, Natalia Jura, and Michael Fischbach for their guidance and for being excellent role models.

I want to thank my parents and grandparents, who always encouraged my love of knowledge and made many sacrifices so that I could attend the best schools when I was growing up. Being able to visit my family at least once a month during my time at UCSF provided an important source of stability that helped smooth out the ups and downs of research.

Last, I want to thank my very patient and loving wife Melissa. Starting our marriage in a tiny apartment with just enough money to pay for rent and food wasn’t exactly what she was expecting! Looking back, this experience brought us closer together and brought a resilience to our marriage that I wouldn’t trade for anything. Mel has always been my greatest cheerleader and my best friend. I’m excited to begin the next chapter of our lives together.
Acknowledgements

Chapters 1, 2, and 3 of this thesis are reprints of the material as it appears in:


Abstract

Drug discovery has historically been most successful by crafting small molecules that mimic metabolites or hormones in order to perturb the function of proteins. However, many of today’s most alluring drug targets are considered “undruggable” according to this prevailing paradigm, because they lack well-defined ligand-binding pockets and instead function through protein-protein or protein-DNA interactions. These challenging drug targets are currently addressed by either directly blocking their interactions with other macromolecules or engaging distant regulatory sites, known as allosteric sites, in order to alter the conformation of the protein. Since high-throughput screening, the most commonly used drug discovery technique, is not often successful at finding ligands that disrupt macromolecular interactions or that bind to allosteric sites, there has been great interest in the development of new methods that enable direct interrogation of the ligand-binding pocket of interest. This thesis begins with a review of the utility of Tethering, a site-directed ligand discovery method, for rapidly generating prototype ligands for protein-protein interfaces (PPIs) and allosteric sites. Chapter 2 describes the adaptation of Tethering to a competitive binding assay format to enable the direct discovery of PPI antagonists. Chapter 3 describes the development of a site-directed high-throughput screen targeting an allosteric peptide-binding site on the protein kinase PDK1. The chemical optimization of these compounds is reported, along with the determination of co-crystal structures to establish their binding modes, and their capacity to inhibit PDK1 signal transduction in cells. Finally, Chapter 4, describes a general computational approach to discover small molecules that target the same PPI in PDK1 via virtual screening. Overall, this thesis provides a compelling case for using site-directed ligand discovery methods to target PPIs. In addition, our work on the protein kinase PDK1 lays the groundwork for new ligand discovery efforts targeting the same allosteric site that is present in numerous other protein kinases.
# Table of Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Site-directed ligand discovery for allosteric sites and protein-protein interfaces</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>FP Tethering: a technique to rapidly identify compounds that disrupt protein-protein interactions</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>A small-molecule mimic of a peptide docking motif inhibits the kinase PDK1</td>
<td>58</td>
</tr>
<tr>
<td>4</td>
<td>Allosteric small-molecule modulators of the protein kinase PDK1 discovered by virtual screening</td>
<td>102</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1-1 ........................................................................................................................................23
Tethering: site-directed ligand discovery

Figure 1-2 ........................................................................................................................................24
Tethering reveals a cryptic allosteric site in caspases-3 and -7

Figure 1-3 ........................................................................................................................................25
Tethering against the cryptic allosteric site in caspase-1

Figure 1-4 ........................................................................................................................................26
Tethering against the cryptic allosteric site in caspase-5

Figure 1-5 ........................................................................................................................................27
Conservation of a cysteine within the cryptic allosteric site across both the inflammatory and executioner caspases

Figure 1-6 ........................................................................................................................................28
Tethering compounds as "chemi-locks" to trap specific caspase conformations for antibody-phage display

Figure 1-7 ........................................................................................................................................29
Tethering against oncogenic K-Ras(G12C) reveals a cryptic druggable pocket near the nucleotide-binding site

Figure 1-8 ........................................................................................................................................30
Tethering to find protein-protein interaction disruptors for the KIX domain of the transcriptional coactivator protein CREBBP

Figure 1-9 ........................................................................................................................................31
Fluorescence Polarization (FP) Tethering, an alternative to mass spectrometry-based experiments, identifies protein-protein interaction disruptors for KIX

Figure 1-10 .......................................................................................................................................32
Tethering against an allosteric site on the kinase PDK1 finds both activators and inhibitors

Figure 1-1 ..............................................................................................................................................33

Tethering to discover ligands for the G-protein-coupled receptor (GPCR) C5AR

Figure 2-1 ..................................................................................................................................................44

Tethering combined with a ligand displacement assay is used to identify chemical probes that disrupt the interaction between KIX and its binding partners

Figure 2-2 ..................................................................................................................................................45

FP- and MS-based Tethering identify overlapping ligand sets for the MLL-binding site.

Figure 2-3 ..................................................................................................................................................46

FP-based Tethering screen identifies ligands that disrupt the interaction between KIX and pKID

Figure 2-4 ..................................................................................................................................................48

Labeling of KIX H602C with 3D4 decreases affinity for both pKID and MLL

Figure 2-S1 ..............................................................................................................................................53

Validation of the FP Tethering competitive binding assay

Figure 2-S2 ..............................................................................................................................................54

Structure-activity relationships for compounds 3D4 and 6D11

Figure 3-1 ..................................................................................................................................................77

Discovery and optimization of diaryl sulfonamides as PIFtide mimics

Figure 3-2 ..................................................................................................................................................79

Structures of the RS compounds bound to the PIF pocket of PDK1

Figure 3-3 ..................................................................................................................................................80

Structural and energetic analysis of the PDK1-PIFtide interaction

Figure 3-4 ..................................................................................................................................................81

Mimicry of PIFtide by diaryl sulfonamide and diaryl dicarboxylate compounds

Figure 3-5 ..................................................................................................................................................82

PIF pocket ligands block S6K1 activation but only weakly affect AKT activation in cells
RS1 enhances the effect of a PDK1 active-site inhibitor to more effectively block both S6K1 and AKT activation.

Figure 3-S1

Optimized FP probe binds to PDK1 with high affinity and yields a large dynamic range.

Figure 3-S2

Diaryl sulfonamides modulate PDK1 through a specific interaction.

Figure 3-S3

Density maps for PDK1-ligand complexes.

Figure 3-S4

Mutation of Leu155 within the PIF pocket of PDK1 confers resistance to the diaryl sulfonamides.

Figure 3-S5

Structural comparison between the PDK1-PIFtide and AKT2-PIFtide complexes.

Figure 3-S6

RS1 selectively modulates the activity of PDK1 but not closely related kinases.

Figure 3-S7

The PIF-pocket ligand RS1 and the ATP-competitive inhibitor GSK combine additively to inhibit the activation of S6K1 in vitro.

Figure 3-S8

Binding of ligands at the ATP-binding pocket and the PIF pocket is not cooperative.

Figure 3-S9

Original images of Western blots.

Figure 4-1

The relative position of the ATP-binding pocket and the helix αC patch on the protein kinase fold.

Figure 4-2

Dose response curves for initial hits 1 and 3 and improved analog 4.
Virtual screening and experimental validation workflow

Figure 4-3 ...........................................................................................................................................120

Figure 4-4 ...........................................................................................................................................122

The crystal structure of PDK1 bound to compound 4

Figure 4-S1 ........................................................................................................................................123

Six PIF pocket models used for virtual screening

Figure 4-S2 ........................................................................................................................................124

Experiments to rule out compound aggregation

Figure 4-S3 ........................................................................................................................................128

The docking pose of compound 4 across the 6 PIF pocket models
List of Tables

Table 2-S1 ......................................................................................................................... 44

Mutation of His602 to Cys in KIX does not disrupt coactivator binding

Table 3-S1 ......................................................................................................................... 100

Data collection and refinement statistics for complexes between PDK1 and diaryl sulfonamides

Table 4-1 ......................................................................................................................... 119

Top virtual screening hits across the six PIF pocket models

Table 4-2 ......................................................................................................................... 119

Structure-activity relationships for the PIF pocket ligand analogs

Table 4-S1 ......................................................................................................................... 125

Data collection and refinement statistics for complexes between PDK1 and diaryl sulfonamides

Table 4-S2 ......................................................................................................................... 129

Data collection and refinement statistics for the complex between PDK1 and compound 4
Chapter 1

Site-Directed Ligand Discovery for Allosteric Sites and Protein-Protein Interfaces

T. Justin Rettenmaier, Sean A. Hudson, and James A. Wells
Introduction

Protein allostery is one of the most prevalent and intriguing mechanisms for regulating protein function.\(^1\) Allostery is mediated through conformational changes where binding at one site remotely affects binding at another. Allosteric ligands can turn proteins on or off, and can even do so by binding at the same allosteric site. For example, the binding of ligands to allosteric sites in GPCRs, nuclear receptors, and enzymes can remotely control their ability to interact with other proteins or substrates, which can result in a wide range of functional outcomes.

Allosteric sites have been of increasing interest in the drug discovery community. Such sites are often less conserved than enzyme active sites that require strict retention and orientation of catalytic and substrate binding sites. Thus, allosteric sites can afford lower hurdles for selectivity, and have the option to either activate or inhibit the target protein. Also, active sites can be challenging when they bind highly charged or large diffuse substrates that are not easily mimicked by drug-like small molecules. Indeed, there are now a number of small molecules developed for allosteric sites in GPCRs, kinases, viral proteins, and many other target classes.\(^2,3\)

However, allosteric sites can be challenging for drug discovery especially when there are no known natural allosteric ligands for the target of interest. Many allosteric interactions involve protein- protein interfaces, and though functional, these sites provide little chemical matter to guide the discovery of drug-like small molecules. In addition, allosteric sites are challenging to annotate, since to do so requires a known ligand or protein to which the target binds and is functionally modulated by. Indeed chemical screening campaigns often reveal new allosteric sites, “orphan sites”, to which no natural effector is known.\(^4\) Protein dynamics and bioinformatics can reveal new cavities not evident in native protein, which may also be opportunities for small molecule drug discovery\(^5,6\), but it is challenging to screen for molecules to bind specifically to
We have developed a site-directed drug discovery technology, called Tethering.\textsuperscript{7} In this approach, a cysteine residue is introduced near a known or putative binding site (Figure 1). The thiol-containing protein is then mixed with a small-molecule fragment (MW 200-300) that contains a disulfide capped with dimethyl-cysteamine.\textsuperscript{8} Each disulfide fragment is allowed to undergo disulfide exchange with the protein in the presence of a redox buffer, typically containing β-mercaptoethanol (β-ME). If the compound binds non-covalently in the vicinity of the cysteine residue, it will preferentially form the mixed protein-fragment disulfide, which is detected by mass spectrometry. This technology was developed at Sunesis Pharmaceuticals and has been applied to over 40 protein targets, with on average 4 cysteine mutants for each target.\textsuperscript{9} Many fragments identified from Tethering have been advanced to free-standing compounds that target proteases\textsuperscript{10,11}, kinases\textsuperscript{12}, and protein-protein interfaces\textsuperscript{13}.

Tethering is ideally suited for discovering and validating allosteric sites. The site-directed aspect allows one to interrogate and discover ligands that engage known or suspected surface sites of interest. Moreover, Tethering can be used to identify “orphan” allosteric sites either from adventitious natural cysteines or engineered ones placed near cavities of interest. Here we describe multiple examples of both. First we will describe work on caspases-1, -3, -5, and -7 where Tethering discovered a natural cysteine at the dimer interface which bound specific tethered compounds that drives each protease to the inactive zymogen-like state. We then review work on K-Ras where an oncogenic cysteine near the active site allowed discovery of tethered inhibitors. Next we describe the use of Tethering to stabilize a dynamic domain of the transcription factor CBP/p300, allowing crystallization and solving of its first X-ray structure as well as generating inhibitors and activators of transcription. Lastly, we focus on two systems for which putative peptide binding sites were known, the kinase PDK1 and the GPCR C5a receptor. In both of these cases there was a modeled peptide allosteric binding site for which we found
tethered compounds, some of which were allosteric activators and others allosteric inhibitors. These studies reveal the power of the Tethering technology to discover small molecule fragments and to identify and mechanistically characterize allosteric sites.

2. Caspases

Caspases are a family of dimeric cysteine proteases that cleave after the Asp residue in a short tetrapeptide (often XXXD) motif. Like many proteases, the caspases are expressed as catalytically inactive zymogens that must be proteolytically activated by an upstream protease. Caspases can be subdivided into two main classes: the inflammatory caspases (-1, -2, -4, -5) and the apoptotic caspases (-3, -6, -7, -8, -9). The apoptotic caspases deconstruct the cell during programmed cell death. In response to apoptotic stimuli, the initiator caspases (-8, -9) are activated and proceed to cleave and activate the executioner caspases (-3, -6, -7). Through the coordinated cleavage of hundreds of different proteins, the executioner caspases trigger many of the hallmarks of apoptosis including membrane blebbing and chromatin laddering.

Caspases represent an important class of drug targets for stroke, ischemia, cancer, and inflammatory diseases. However, despite their biological significance, no caspase-directed therapies are currently available. The active sites of caspases have been difficult to target with drug-like compounds owing to their preference for negatively charged substrates. Recently, several successful Tethering campaigns have identified novel allosteric inhibitors of caspases.

2.1 Tethered allosteric inhibitors of executioner caspases-3 and -7

In 2004, Hardy et al. of Sunesis Pharmaceuticals reported the first Tethering screen against caspases. A library of 10,000 disulfide fragments was screened against endogenous cysteines of wild-type active caspase-3. Active caspase-3 contains five surface-exposed cysteines in addition to the catalytic cysteine. Two compounds were discovered, named FICA and DICA,
which completely inhibited caspase-3 activity following 100% covalent modification of a single
cysteine (Figure 1-2a). MS analysis revealed that the modified cysteine was not the catalytic
cysteine but a cysteine present in the small subunit. Remarkably, this allosteric cysteine was
also present in the executioner caspase-7, and FICA and DICA also quantitatively inhibited
active caspase-7. Labeling of the allosteric cysteine with FICA/DICA was mutually exclusive
with labeling of the active site cysteine with the peptide Z-DEVD-fluoromethylketone,
suggesting that the binding of FICA/DICA induced a large conformational change that was
incompatible with substrate binding.

An X-ray crystal structure of FICA- or DICA-modified caspase-7 showed the allosteric inhibitors
conjugated to Cys290 in a central deep cavity at the caspase dimer interface (Figures 1-2b,c).
Despite being 14 Å from the active site, this site functionally couples to the active site and
prevents substrate binding. FICA forms two intermolecular hydrogen bonds with itself across the
dimer interface, and each FICA molecule displaces Y223. When comparing to the structure of
active caspase-7 bound by substrate-mimic DEVD, we see that DEVD forces Y223 and
adjacent R187 down (Figure 1-2d). FICA and DICA force Y223 and subsequently R187 into the
up position by steric exclusion. In the procaspase-7 zymogen structure, R187 is also up and
Y223 is free to assume either the up or the down conformation. Hence, the FICA and DICA
allosteric inhibitors appear to trap a zymogen-like conformation. The inhibitors also induce
conformational changes particularly in substrate-binding region loops into zymogen-like
conformations (not illustrated). Subsequent mutational analysis further evaluated an allosteric
“switch” that connects the allosteric and active sites.16,17

2.2 Tethering inflammatory caspase-1

To probe the generality of this dimer interface site, Scheer et al performed a Tethering screen
against human inflammatory caspase-1.18 Caspase-1 is primarily responsible for processing and
activating the inflammatory cytokine interleukin 1β. Despite sharing only a 20-30% amino acid sequence identity with the apoptotic caspases, caspase-1 also contains a cavity at the dimer interface with a cysteine (Cys331) in a position nearby to those in caspase-3 and -7.

A library of ~8,000 disulfide fragments was screened by mass spectrometry and then an in vitro enzymatic assay, which led to the identification of the thienopyrazole compound 34 (Figure 1-3a). The caspase-1 mutant C331A was neither labeled nor inhibited by compound 34. A crystal structure of caspase-1 in complex with compound 34 shows two molecules of the allosteric inhibitor trapped to Cys331 in the central cavity at the dimer interface (Figures 1-3b,c). The compound-binding site is 15Å away from the active site yet these sites appear to be functionally coupled much like the FICA/DICA- binding site in caspases-3 and -7. Binding of compound 34 causes a large conformational change wherein Arg286-Glu390 (located next to the catalytic cysteine) break a salt bridge and shift >12 Å to interact with the compound. A comparison of the global structures of caspase-1 in the allosterically inhibited or apo states revealed that compound 34 traps an inactive conformation that pre-exists in the ligand-free enzyme. Subsequent mutational analysis revealed a clear allosteric circuit that connected the allosteric and active sites similar to that described for caspase-7.19

2.3 Tethered allosteric inhibitors of caspase-5

More recently, Gao and colleagues extended Tethering to target caspase-5.20 Caspase-5 is also an inflammatory caspase, like caspase-1, but its specific cellular roles remain unclear. Selective inhibitors would be helpful to dissect the role of caspase-5 in the innate immune response. From a screen of ~15,000 disulfide fragments, the naphthyl-thiazole compound 8 was the top hit (Figure 1-4). Compound 8 inhibited 50% of caspase-5 activity at a concentration of 1 µM. Labeling of caspase-5 by compound 8 could be reduced to 50% by 2.3 mM β-ME, which indicated high conjugation strength as this value was 23-fold more stringent than that used
during the primary screen. Compound 8 selectively labeled the allosteric cysteine on the subunit at the caspase-5 dimer interface, but did not label or inhibit caspase-1. This selectivity is remarkable considering the high degree of sequence conservation between these inflammatory caspases (59% identity and 71% similarity in the catalytic subunits).

2.4 General allosteric regulation at the caspase dimer interface

The success of Tethering against the allosteric cavity located at the dimer interface of caspases-1, -3, -5 and -7, suggests that this regulatory site is common between inflammatory and apoptotic caspases. This possibility inspired mutational studies to further define the residues that functionally couple the allosteric and active sites in both caspase-1 and caspase-7.\(^{16,19}\) The deadly nature of caspases to cells has necessitated the evolution of many levels of regulation to ensure their activity only at the appropriate times. Thus, it is tempting to speculate that the central cavity may serve as a regulatory site for an as yet undiscovered effector molecule that acts as an additional level of native regulation. This allosteric cavity featuring a natural cysteine appears across most active caspases (Figure 1-5). The sequence diversity at the allosteric site across the caspases may enable the future development of selective small-molecules inhibitors for each family member.

2.5 Using disulfide fragments as “chemi-locks” to generate conformation-specific antibodies

The dynamic conformational states of caspase-1 (active, allosterically inhibited and ligand-free) present potential antigens that could be used to find antibodies that selectively trap each conformation (Figure 1-6). Conformation-specific antibodies would have utility in probing the equilibrium distribution of caspase-1 conformational states both \textit{in vitro} and in cells. Gao and colleagues from UCSF and Genentech, used covalent ligands as “chemi-locks” to homogeneously trap caspase-1 into one of two alternative conformations.\(^{21}\) The covalent active-
site inhibitor (Ac-YVAD-chloromethylketone) locked the on-state, and the disulfide fragment 34 (Figure 1-3a) was used to lock the off-state. These conformation-trapped forms of the protease were then used as antigens in alternating rounds of selection and anti-selection for antibody fragments (Fabs) displayed on phage (Figure 1-6). After affinity maturation, two high-affinity Fabs were isolated with \( K_d \) values ranging from 2-5 nM, and each bound to their cognate conformer 20- to 500-fold more tightly than the opposite conformational state.

The Fabs were able to activate (Fab\(_{\text{on}}\), \( EC_{50} = 11.7 \text{ nM} \)) or inhibit (Fab\(_{\text{off}}\), \( EC_{50} = 920 \text{ nM} \)) caspase-1 in an \textit{in vitro} proteolysis assay. When converted into full-length human IgGs, both bound to ligand-free caspase-1 like their Fab counterparts. IgG\(_{\text{off}}\) inhibited caspase-1-mediated processing of interleukin-1β in monocyte (THP-1) cell lysates. The IgG\(_{\text{on}}\) monoclonal antibody was used to probe and localize active conformers of caspase-1 in THP-1 cells, showing that activated caspase-1 is concentrated in a central structure in the cytosol, similar to what has been described as the pyroptosome. In all, the approach provides a general strategy for producing multiple conformation-specific antibodies to proteins.

\textbf{3. Tethering K-Ras(G12C)}

One-third of all tumors harbor mutations in Ras genes, but GTPases encoded by these mutant genes have eluded targeting by therapeutic agents.\(^{22}\) Ras serves as an important switch for cell signaling, critical to cell cycle progression.\(^{23}\) Ras cycles between the GTP-bound active and GDP-bound inactive signaling states. Oncogenic mutations to K-Ras such as G12C impair its ability to hydrolyze GTP to GDP. With diminished GTPase activity, the GTP-bound active state of Ras accumulates, which leads to unregulated signaling and ultimately tumorigenesis.

Drug discovery efforts have long attempted to halt unregulated Ras, but have failed to identify potent and selective small molecules. GTP and GDP are present in the cell at millimolar concentrations and bind to Ras with picomolar affinity, which renders the active site of Ras
virtually intractable for inhibitor development. Drug development thus turned to inactivating RAS indirectly through the development of farnesyltransferase inhibitors, but these drugs had limited efficacy in clinical trials.24 Attention has recently focused on inhibitors of the prenyl-binding protein PDEδ, as correct subcellular localization and signaling by farnesylated K-Ras is regulated by binding with PDEδ.25

Ostrem et al. recently reported a new Tethering approach specifically targeting the K-Ras(G12C) mutant, which is found in a substantial proportion of lung cancers.26 A library of 480 disulfide fragments were screened for binding the oncogenic cysteine mutant K-Ras(G12C) in the GDP-bound inactive state. Disulfide fragment 6H05 gave the greatest degree of modification (94%; Figure 1-7a). Compound 6H05 did not label wild-type K-Ras, which contains three endogenous cysteine residues. The labeling of K-Ras(G12C) by 6H05 was not diminished by high concentration 1mM GDP, which suggested that the compound bound to a site that did not overlap with GDP. However, pre-loading of K-Ras(G12C) with GTP significantly impaired the binding of 6H05.

A small number of analogs of 6H05 were made to increase its affinity and the top analog 6H05-2 was 4.2-fold more potent (Figure 1-7a). A 1.3 Å co-crystal structure of 6H05-2 bound to K-Ras(G12C) in the GDP state revealed that 6H05-2 does not bind in the nucleotide pocket, but rather extends from Cys12 into a previously unobserved binding pocket composed largely of switch-II, called the switch-II pocket (Figure 1-7b). The switch-I and switch-II loops intimately interact with the nucleotide-binding pocket of K-Ras and mediate interactions with a multitude of downstream effectors in a GTP-dependent manner. The switch-II pocket is not visible in other structures of K-Ras, and thus it is probably highly dynamic when GDP is bound. In the GTP-bound active state conformation of RAS, residues from switch-II entirely fill the switch-II pocket. Thus, compound 6H05-2 binds to a cryptic allosteric pocket and displaces switch-II but has little effect on switch-I (Figure 1-7c).
Since disulfide fragments are susceptible to reduction in cells, the tethering hits were converted to irreversible cysteine-reactive electrophiles, including acrylamides and vinyl sulfonamides. The fragments were chemically optimized through the generation of 100 analogs, resulting in electrophiles 8 and 12 that efficiently labeled K-Ras(G12C) at 10 µM (Figure 1-7d). Treatment with electrophile 12 modified K-Ras(G12C) but not BSA or wild-type K-Ras, indicating the electrophile did not promiscuously label cysteines but selectively reacted with K-Ras(G12C). The covalent bond between 12 and G12C was essential for binding, as the naked fragment without its electrophile did not prevent labeling of K-Ras(G12C) by 12.

The authors first assessed the functional consequences of labeling K-Ras(G12C) using an in vitro nucleotide binding assay. Labeling K-Ras(G12C) with 8 or 12 decreased its affinity for GTP relative to GDP by nearly 4-fold. Based on this result, they expected that treating K-Ras with these compounds would lead to the accumulation of its GDP-loaded inactive state. Moreover, the conformational rearrangements to K-Ras were predicted to be incompatible with binding to downstream effectors. Indeed, treatment of K-Ras(G12C) mutant lung cancer cells with 12 diminished the interaction between K-Ras and its binding partners B-RAF and C-RAF.

Excitingly, treatment of a panel of lung cancer cell lines with 12 led to apoptosis only in those cells annotated to harbor the K-Ras(G12C) mutation. In all, the lead electrophile compound needs additional affinity optimization and evaluation in vivo, but Ostrem et al. provide a good starting point for new drug discovery efforts targeting K-Ras(G12C) and its cryptic allosteric switch-II pocket.

4. The master transcriptional coactivator CREB-binding protein

Small molecules that directly modulate transcriptional regulators promise direct control of gene expression by bypassing upstream regulatory pathways. However, with the exception of steroid hormone receptors and histone modifying enzymes, most transcriptional regulators have eluded
modulation by small molecules due to the chemical intractability of their highly charged DNA-binding sites and extended protein-protein interfaces. CREB-binding protein (CBP), also known as p300, is a master transcriptional coactivator that serves as a transcriptional hub in all metazoans. CBP interacts with numerous transcriptional activators using several different domains. The KIX domain of CBP is a 90-residue three-helix bundle that interacts with at least 10 different transcriptional activators at two distinct binding sites (Figure 1-8a). Interestingly, the two binding sites exhibit positive allosteric coupling where the binding of a transcriptional activator at one site enhances the binding of a second activator at the other site. Unfortunately, the dynamic nature of the KIX domain has complicated in-depth analyses of its structure and the allosteric coupling between its binding sites. Using Tethering, Mapp and colleagues have identified fragments that act as pharmacological chaperones to stabilize the protein for structure/function studies. Excitingly, many of these fragments inhibit the binding of transcriptional activators to one or both binding sites on the KIX domain of CBP.

4.1 Tethering to find stabilizers of the KIX domain of CBP

In 2013, Wang et al. reported a Tethering screen against the KIX domain of CBP with the aim of identifying fragments to stabilize KIX and facilitate crystallographic analysis. The authors introduced a cysteine mutation at L664 of the KIX domain, which lies adjacent to the known binding site for the transcriptional activation domain of mixed-lineage leukemia protein (MLL) (Figure 1-8a). A screen of 480 disulfide fragments yielded two hits, 1-10 and 2-64, which strongly tethered to the KIX domain (Figure 1-8b). These fragments increased the melting temperature of KIX by 15-18°C, protected 40% and 55% of amides from hydrogen-deuterium exchange, and led to a 5-fold and 35- fold increased resistance to degradation by the protease thermolysin. Lastly, both fragments bound to KIX competitively with MLL. Altogether, these results suggested that these covalent small molecules dramatically stabilized this highly dynamic protein by occupying the MLL-binding site. Ultimately, labeling KIX with disulfide
fragment 1-10 enabled its crystallization and the determination of the first KIX crystal structure at 2.0 Å resolution (Figure 1-8c). This study revealed the utility of disulfide fragments as small-molecule stabilizers for enabling the structural characterization of highly dynamic proteins.

**4.2 Dissecting the allosteric coupling between binding sites on KIX**

Wang and colleagues next sought to understand the mechanism of allosteric communication between the two binding sites on KIX. In principle, the allosteric coupling between KIX’s two binding sites could be explained by changes in the rate of formation of a ternary complex or the rate of its dissociation. To discriminate between these possibilities, the authors performed stopped-flow binding experiments to measure the rates of binding ($k_{on}$) and dissociation ($k_{off}$) for a fluorescent peptide (pKID) when mixed with various liganded states of KIX. These experiments required prior formation of a complex between KIX and a ligand at the MLL-binding site, so that binding at the pKID-binding site could be observed. Using the covalent tool compound 1-10, KIX was quantitatively labeled and trapped into a state of positive allosteric coupling (modification of N627C) or a state of negative allosteric coupling (modification of L664C). Surprisingly, the kinetic binding experiments revealed that the basis of allosteric coupling between KIX’s two binding sites could be explained solely by modulation of the dissociation rate ($k_{off}$) of the ternary complex. That is, positive allosteric modulators stabilized the ternary complex and resulted in a slower off-rate, whereas negative allosteric modulators had the opposite effect. These findings may extend to understanding binding cooperativity in the context of other protein-protein interactions that involve intrinsically disordered domains like the transcriptional activation domains of MLL and pKID studied here. Given the ease of identifying Tethering fragments, mechanistic studies of this type could be initiated in many other systems.

**4.3 Rapid identification of pKID-competitive fragments for KIX**

Despite Tethering against cysteine residues placed directly adjacent to the MLL-binding site in
the KIX domain (Figure 1-8a), Mapp and colleagues remarked that a large fraction of fragments discovered from these screens did not efficiently disrupt the interaction between KIX and MLL. Thus, in order to select only those fragments that are effective PPI inhibitors, Tethering was adapted to a competitive binding assay where the disruption of the PPI would be monitored by fluorescence polarization (Figure 1-9). To create displaceable binding probes, the authors conjugated fluorophores to the peptide ligands MLL, pKID, and c-Myb. As a proof of concept, they showed that this new approach, termed FP Tethering, could correctly identify known MLL-competitive fragments against KIX N627C. Next, they screened 960 disulfide fragments against KIX H602C to find ligands that could displace pKID and c-Myb on the opposite face of the protein. The screen was performed at low, medium, and high stringency (0.2 mM, 1 mM, and 5 mM β-ME) to expedite the identification of the most potent fragments all at once. The authors selected the top 10 of 63 hits (6.6% hit rate) to validate by the traditional mass spectrometry readout. 9 of the 10 hits were confirmed, including the extremely efficient ligand 3D4 (β-ME50 >5 mM). Surprisingly, 3D4 not only impeded the binding of pKID (6.5-fold) and c-Myb (9-fold) but also MLL (9-fold), which binds on the opposite face of the KIX domain. The authors are further pursuing covalent analogues of 3D4 for use in studying CBP/p300 biology.

5. Tethering against the PIF pocket of Phosphoinositide-Dependent Kinase 1 (PDK1)

The critical role of protein kinases as regulators of cell state has fueled enormous interest over the past 15 years to develop kinase inhibitors as chemical probes and therapeutics. Nearly all kinase inhibitors target the highly conserved ATP-binding pocket, and as a consequence these molecules usually inhibit many kinases simultaneously. These off-target effects diminish the value of chemical tools and can cause undesirable side effects in patients. To address these limitations, significant effort has focused on targeting allosteric sites distinct from the ATP-binding pocket. Several allosteric kinase inhibitors are now in clinical trials, and many more are being developed, but the discovery of chemically tractable allosteric sites remains a major
bottleneck in the development of new compounds.

The protein kinase PDK1 is a member of the AGC family of serine/threonine kinases, which collectively regulate aspects of cell growth, proliferation, metabolism, and programmed cell death. PDK1 controls the activation of at least 23 other AGC kinases, including the oncogenic kinases AKT and S6Ks. PDK1 has therefore been targeted by numerous drug discovery programs, but potent and highly selective inhibitors have had limited anticancer efficacy. In order to overcome the limitations of PDK1 inhibitors, recent efforts have focused on targeting the PDK1-interacting fragment (PIF) pocket of PDK1, which is an allosteric peptide-docking site that PDK1 uses to recruit its substrates.

Sadowsky et al. used Tethering to probe the structure, chemical tractability and functional plasticity of the PIF pocket of PDK1. We first mapped the interaction between the PIF pocket and a known peptide ligand PIFtide through peptide disulfide-trapping experiments. Six residues lining the PIF pocket were individually mutated to cysteine (Figure 1-10a) and the efficiency of disulfide formation was measured for each mutant across a panel of cysteine-mutant PIFtides. Next, the crosslinking frequencies were converted to distance restraints and were used to create a structural model that suggested how hot spot residues within PIFtide engage the PIF pocket of PDK1. This structural model closely mimics a recent crystal structure of the PDK1-PIFtide complex, validating the disulfide- crosslinking approach.

Next, we explored whether disulfide fragments could be identified that mimic the PIFtide and what range of effects such fragments would exert on PDK1’s catalytic activity. A library of 480 disulfide fragments was screened against each of the 6 cysteine mutants of PDK1 under stringent conditions (10 mM β-ME). The hit rate varied widely from 1.3% for I119C to 13.8% for K115C. Remarkably, these disulfide fragments revealed a large degree of functional plasticity at the PIF pocket: both activators and inhibitors could be found at every cysteine position. The
widest range of functional effects was observed for T148C spanning a 19-fold difference in catalytic activity (32% to 630%; Figure 1-10b). To understand the structural basis of these allosteric effects, we solved crystal structures of PDK1 T148C in complex with activating or inhibiting fragments. The best activator JS30 (Figure 1-10c) enforced a closed conformation of the αC helix and correctly positioned residues key to catalysis. In contrast, the inhibitory fragment 1F8 held the αC helix in an open conformation in which the ATP-binding pocket is unliganded and catalytic residues are out of register (Figure 1-10d). These studies revealed that different ligands binding to the same allosteric site can trap a range of conformations between full on and off-states. Moreover, this work established a number of chemical starting points for the further development of allosteric PDK1 inhibitors that might overcome the limitations of existing active-site inhibitors.

6. Tethering against GPCRs: Complement 5A Receptor

G-protein coupled receptors (GPCRs) are transmembrane receptors that sense a multitude of extracellular signals including hormones, neurotransmitters, odors, and even photons. Given their chemical tractability and central roles in numerous cellular processes, GPCRs are the target of nearly 40% of all approved drugs.\textsuperscript{36} Despite the intense focus of the pharmaceutical industry on GPCRs, there is still much we have to learn about this protein family. For example, knowing that two drugs bind to the same site on a GPCR tells us nothing about whether each drug activates or inhibits the receptor. Moreover, we have very limited structural information for the vast majority of GPCRs, due in part to a lack of stabilizing ligands. New technologies to rapidly identify GPCR ligands could provide tool compounds that enable the mechanistic and structural studies needed to address these questions.

In 2005, Buck and colleagues reported two studies that extended the scope of Tethering from beyond soluble proteins to targeting a GPCR, the Complement 5a Receptor (C5aR).\textsuperscript{37,38} C5aR
binds to a 74-amino acid protein called C5a, which is a highly inflammatory molecule that mediates the pathology of sepsis and several autoimmune disorders. Medicinal chemistry efforts in the early 1990s showed that 6-mer peptide analogs derived from the C-terminus of C5a can bind to C5aR and either activate or inhibit the receptor.\(^{39}\) In the first study, the first 3 amino acids of these 6-mer peptides were removed, which destroyed their ability to bind. However, when the truncated peptides were appended with a cysteine residue they could tether to specific cysteines introduced into the C5aR and signal (Figure 1-11a,b). Much like PDK1 it was possible to activate or inhibit the receptor by conjugating different disulfide ligands to the same cysteine.

In a follow-up study, Buck and Wells used Tethering to identify non-peptidic fragments that agonize and antagonize C5aR. Since mass spectrometry was not suitable to monitor disulfide formation with this transmembrane receptor, the authors instead adapted a radioligand displacement assay to identify fragments that bind competitively with C5a. To prepare the four C5aR mutants for Tethering, the authors expressed each mutant in COS-7 cells and prepared membranes by hypotonic lysis and centrifugation through a sucrose cushion. Next, they screened 10,000 fragments in pools of 10 to identify fragments that diminished the binding of \(^{125}\)I-C5a by >15%. The hit rates ranged from 0.2% to 0.7% for the three cysteine mutants that were predicted to be within range for disulfide formation. No hits were identified for the control mutation F93C, which was predicted to be 10-15 Å from the ligand-binding site (Figure 1-11a,b). Remarkably, the disulfide ligands could label C5aR mutants expressed on the surface of living cells and activate or inhibit C5aR as read out by IP3 accumulation. Importantly, the effects of the disulfide fragments were stereospecific, as compound 14, the enantiomer of the active compound 6, had minimal effect on C5aR signaling (Figure 1-11c,d).

Overall these studies demonstrated that Tethering is suitable for rapidly identifying ligands for membrane proteins. This approach could be particularly powerful for generating chemical tools
that enable the mechanistic study of the basis of agonism versus antagonism or covalent ligands that stabilize receptors to enable structural characterization. Perhaps most excitingly, this approach could be exploited to explore the function of the nearly 150 “orphan” GPCRs that have no known ligands.

7. Conclusions and Future Directions

These studies exemplify the power of Tethering to discover ligands to known and orphaned allosteric sites. The caspases show that disulfide fragments can be found for natural cysteines that lock proteins in transient off-conformations even when the protein is predominantly in the on-state. The K- Ras case similarly used a natural cysteine to discover disulfide fragments that bind to a new site not evident in previous structures. The case of KIX shows that engineered cysteines can be used to discover disulfide fragments that stabilize a dynamic protein to enable crystallization and structural analysis. The case of PDK1 shows that Tethering against a known allosteric site can reveal a wider range of possible functional effects than were appreciated from studying natural ligands. Lastly, the case of the C5a receptor shows that Tethering can target GPCRs and potentially other membrane proteins to identify both agonists and antagonists.

Tethering is amenable to many screening paradigms. Most of the cases described here used mass spectrometry to detect the mixed disulfide between a purified protein and a disulfide fragment. This mass spectrometry approach can be applied to single compounds or pools of 5-10 compounds provided they differ in mass sufficiently to be easily resolved. Importantly however, mass spectrometry is not required to perform Tethering. The cases of KIX and C5aR show how functional assays like fluorescence polarization and radioligand displacement can be used to detect disulfide fragments with the desired functional effect. In unpublished work, we have also performed Tethering screens using enzymatic assays on GTPases and proteases. More broadly, Tethering could be combined with any functional or biophysical assay that is
compatible with disulfide-containing compounds and β-mercaptoethanol, increasing the accessibility of this powerful technology.

Tethering has unique advantages over other fragment screening approaches that use X-ray, NMR or SPR. While fragments often bind with high ligand efficiency, their low affinities for the target protein can make it challenging to assay their functional impact or to saturate the binding site for structural analysis. Without knowing the binding mode or functional impact of a fragment, efforts to derivatize the compounds or understand the function of the putative allosteric site are extremely challenging. In contrast, the covalent nature of the disulfide fragments used in Tethering allows one to readily generate homogenous protein-fragment conjugates for structural and functional analysis. While the use of a covalent bond during the Tethering screen reduces the degrees of freedom for binding, the fragments discovered by Tethering have comparable non-covalent binding affinities to fragments discovered by other means (0.1 to 5 mM). In addition, it is simple to derivatize disulfide fragments and monitor their SAR by determining the 50% conjugation point in the presence of increasing β-ME (often shifting from 0.5 to >10mM) or increasing compound concentration (typically 1-50µM in 0.5mM β-ME). Although one can readily advance the optimized disulfide fragment in non-covalent mode just like other fragment approaches (IL-240 and caspase-310), it is also possible to convert the disulfide into a covalent electrophile for intracellular work as was shown for K-Ras.

In addition to its utility as a drug discovery technique for challenging targets, Tethering is also a very powerful approach for studying the mechanisms of allostery. In virtually every example, we could trap proteins in active or inactive states and determine their structures. We could also use these “chemi-locked” proteins to develop conformation-specific antibodies that were useful for tracking enzyme activation in cells. Allostery is a fascinating property of protein dynamics and Tethering is ideally suited to generate useful fragments for understanding and exploiting it for drug discovery.
References


24. Rowinsky EK (2006) Lately, it occurs to me what a long, strange trip it's been for the


the protein kinase PDK1. Proc Natl Acad Sci USA 111(52):18590-18595.


Figure 1-1. Tethering: site-directed ligand discovery. A putative ligand-binding site on the protein of interest is directly targeted for ligand discovery by screening a library of disulfide-containing compounds against a native cysteine or engineered cysteine near the site of interest. Small molecules that bind favorably to the protein form a stable disulfide adduct that resists reduction by β-mercaptoethanol. The percentage of the protein labeled by the small molecule is readily determined by intact protein mass spectrometry and serves as a proxy for the molecule’s potency.
Figure 1-2. Tethering reveals a cryptic allosteric site in caspases-3 and -7. a. Chemical structure of FICA and DICA. b. Active caspase-7 dimer central cavity (red) with C290 labeled. The active site is colored green. c. Crystal structure of caspase-7 bound by DICA. d. Conformations of residues Y223 and R187 for active caspase-7 bound to substrate-mimic DEVD (left), caspase-7 bound to allosteric inhibitor DICA (middle), or zymogen caspase-7 (right).
Figure 1-3. Tethering against the cryptic allosteric site in caspase-1. 

a. Chemical structure of compound 34. 
b. Crystal structure of caspase-1 bound to compound 34 at the dimer interface. 
c. Close-up view of the ligand-binding site.
Figure 1-4. Tethering against the cryptic allosteric site in caspase-5. Chemical structure of the top hit compound 8.
Figure 1-5. Conservation of a cysteine within the cryptic allosteric site across both the inflammatory and executioner caspases. Sequence alignment of the small subunits of the human executioner caspases-3, -6, and -7 and inflammatory caspases-1, -4, and -5. Cysteines are colored in orange. A structural overlay reveals that Cys264 of the executioner caspases occupies a position within the cryptic site that is similar to Cys331 of the inflammatory caspases.
Figure 1-6. Tethering compounds as “chemi-locks” to trap specific caspase conformations for antibody-phage display. A covalent peptide substrate or the allosteric Tethering inhibitor were used to lock caspase-1 into different conformations for antibody generation via phage display. The caspase-antibody complexes shown on the right are models.
Figure 1-7. Tethering against oncogenic K-Ras(G12C) reveals a cryptic druggable pocket near the nucleotide-binding site. **a.** Chemical structures of compound 6H05 and analog 6H05-2. **b.** Co-crystal structure K-Ras(G12C) labeled by 6H05-2 (cyan) and bound to GDP (grey) and Ca\(^{2+}\) (green). **c.** Structural basis of K-Ras(G12C) modulation by disulfide fragments. On GTP hydrolysis (top to bottom left), removal of the γ-phosphate leads to relaxation of the spring-loaded active Ras-GTP state. Drug binding (bottom right) moves switch-II even further away and interferes with GTP binding. **d.** Chemical structures of the optimized electrophiles 8 and 12.
Figure 1-8. Tethering to find protein-protein interaction disruptors for the KIX domain of the transcriptional coactivator protein CREBBP. a. The two binding surfaces on opposite faces of the KIX domain (grey) are shown bound to MLL in magenta and pKid in cyan [28]. Tethering has been conducted at all positions marked with red spheres. b. Chemical structures of top hits from a Tethering screen against KIX L664C. c. Crystal structure of KIX L664C in complex with disulfide fragment 1-10.
Fluorescence Polarization (FP) Tethering, an alternative to mass spectrometry-based experiments, identifies protein-protein interaction disruptors for KIX.

a. Premise of FP Tethering. Disulfide formation is detected by monitoring displacement of a known ligand that has been appended with a fluorophore. b. Chemical structure of the disulfide fragment 3D4, which labels KIX H602C and binds to the pKID pocket of KIX.
Figure 1-10. Tethering against an allosteric site on the kinase PDK1 finds both activators and inhibitors. a. Residues lining the PIF pocket of PDK1 that were mutated to cysteine are shown as red spheres. b. Chemical structures of the best activator JS30 and the best inhibitor 1F8. c, d. Crystal structures of PDK1 T148C in complex with the activator JS30 or the inhibitor 1F8. The αB helix, αC helix, and β4/5 strands that form the PIF pocket are colored pink, yellow, and cyan, respectively.
Figure 1-11. Tethering to discover ligands for the G-protein-coupled receptor (GPCR) C5AR. a, b. Placement of cysteine mutations within the C5aR peptide-binding pocket. Thiol-to-thiol distances between C5aR and a peptide ligand are shown in dotted grey lines. F93 is predicted to be >10Å away from the ideal disulfide distance of 2.4Å. c. Chemical structure of agonist compound 6. d. Compound 6 activates C5aR G262C more than C5a does, but its enantiomer compound 14 is much less active.
Chapter 2

FP Tethering: a technique to rapidly identify compounds that disrupt protein-protein interactions.

T. Justin Rettenmaier*, Jean M. Lodge*, James A. Wells, William Pomerantz, and Anna Mapp
Abstract

Tethering is a screening technique for discovering small-molecule fragments that bind to predetermined sites via formation of a disulfide bond. Tethering screens traditionally rely upon mass spectrometry to detect disulfide bind formation, which requires a time-consuming liquid chromatography step. Here we show that Tethering can be performed rapidly and inexpensively using a homogenous fluorescence polarization (FP) assay that detects displacement of a peptide ligand from the protein target as an indirect readout of disulfide formation. We apply this method, termed FP Tethering, to identify fragments that disrupt the protein-protein interaction between the KIX domain of the transcriptional coactivator CBP and the transcriptional activator peptide pKID.
Introduction

Protein-protein interactions (PPIs) underpin all cellular processes, and dysregulation of PPI networks is strongly correlated with human disease. For this reason, synthetic molecules that modulate PPIs are highly sought tools.\(^1\) Despite their importance, small molecule PPI modulators are difficult to obtain through either screening or design.\(^2\) This is largely due to the intrinsic features of PPIs, most of which are comprised of significantly larger surface area (average \(\sim 1949 \pm 760 \, \text{Å}^2\)) than typical protein-ligand interfaces and are often flatter with few interaction features.\(^3\) Indeed, PPIs have often been described as ‘undruggable’ due to the challenges associated with identifying small molecules that can effectively engage these binding interfaces.

Tethering is a screening strategy that circumvents many of the difficulties associated with PPIs and has been successfully used to discover a range of small molecule modulators.\(^4,5\) It is a fragment discovery method in which a library of disulfide-containing fragments (MW <300 Da) are screened under reversible conditions against a protein target bearing a native or engineered cysteine adjacent to the binding site of interest.\(^6,7\) Fragments that interact favorably with the protein target bias the equilibrium towards the mixed disulfide, which is subsequently detected by liquid chromatography-mass spectrometry (LC-MS) (Figure 2-1a). The resulting fragment molecules can be converted to non-covalent inhibitors by growing the fragment or used as covalent binders for functional and biophysical studies.\(^8\)

We recently reported the application of Tethering to discover small molecule ligands for the KIX domain of the master coactivator CBP/p300.\(^9\) Several fragments identified from this screen proved to be excellent inhibitors of the PPI formed between KIX and the transcriptional activator MLL as well as enhancers of the complex that KIX forms with the transcriptional activation domain of CREB (pKID). Nonetheless, we noted that some of the fragments that bound to KIX efficiently did not affect KIX PPIs. In retrospect this is not surprising since the readout of the
screen is binding, not function. We hypothesized that combining Tethering with a competitive inhibition readout would provide a more direct route to PPI modulators (Figure 2-1c). Here we show that fluorescence polarization (FP) Tethering is a rapid method for the direct discovery of PPI modulators and use this method for the identification of effective inhibitors of the complex formed between KIX and pKID.

Results and Discussion

Fluorescence Polarization (FP) is a homogenous, mix-and-read method used to directly measure the fraction of a small fluorescently-labeled peptide tracer that is bound to a larger protein. Binding of the tracer to the protein results in high fluorescence polarization, but the tracer’s fluorescence is rapidly depolarized following displacement by an unlabeled ligand (Figure 2-1a). This type of ligand displacement assay provides a way to measure the affinity between the protein target and an unlabeled ligand. However, FP ligand displacement assays are less sensitive than x-ray crystallography or NMR for detecting the low affinity interactions of fragments, which typically occur in the hundreds of micromolar to millimolar range. Since Tethering of fragments via a disulfide bond enhances their affinity 10- to 100-fold, we reasoned that these binding events should be detectable by FP.

To test this approach we first used the results from our earlier Tethering screen against cysteine mutants of the KIX domain. The KIX domain is a small (90 residue) three-helix bundle containing two binding sites that can be occupied simultaneously by different transcriptional activators (Figure 2-1b). These two binding sites are in allosteric communication, with the binding of MLL in the smaller and deeper of the two sites leading to up to 2-fold enhancement of binding with activators such as pKID (the transcriptional activation domain of CREB) at the second, more shallow binding site. Two disulfide fragments isolated from the original Tethering screen bind well to a N627C mutant of KIX, fragments 1A10 and 1C11, and also were
effective inhibitors of MLL binding when covalently associated with KIX. Rather than measuring binding inhibition using a fully-labeled protein, we reasoned a competitive inhibition readout could report on fragment-labeling of the KIX N627C mutant during the Tethering reaction. Indeed, in the presence of 5 mM β-ME, 82% of the KIX N627C mutant was labeled in the presence of 250 μM of 1A10, and the inhibition of a fluorescently-labeled MLL peptide was observed in a dose-dependent fashion as a functional consequence of protein labeling (IC50 68 μM, Figure 2-S1) as a functional consequence of protein labeling. The ability to increase or decrease percent inhibition levels by changing the β-ME concentration affords a control for ruling out fluorescent impurities or other false positives.

Next, 80 disulfide-containing fragments were screened at two different β-ME concentrations against the KIX N627C mutant (Figure 2-2b). The FP Tethering screen identified the same set of ligands that were previously identified in the MS-based screen (Figure 2-2c). Notably the hits that resulted in the largest inhibition of the KIX domain and MLL interaction were also the most potent hits from our MS-based screen (data not shown). These results suggested that the FP Tethering assay was suitable for high-throughput identification of fragments that disrupt the interaction between KIX and its coactivators.

To test the FP Tethering approach in a pure discovery mode, we targeted the second binding site within the KIX domain, which is used by pKID in addition to the activator c-Myb. This is the considerably more challenging of the two sites, with a larger surface area (1480 Å²) and a less defined surface. In preparation for the screen, we mutated several residues lining the binding site to cysteine and determined the affinity of the mutant KIX domains for both pKID and c-Myb tracers (Table 2-S1). The KIX H602C mutant retained its affinity for both tracers and it was thus chosen for the FP Tethering screen. The competition assay was developed with 25 nM FITC-labelled pKID and 4 μM of KIX H602C. These conditions were sufficient to detect displacement of FITC-labelled pKID tracer from the KIX domain by the unlabelled pKID peptide.
With these assay conditions in hand, we screened 960 disulfide-containing fragments at three different β-ME concentrations against a fluorescent pKID tracer in complex with KIX H602C (Figure 2-3a). Fragments that yielded total fluorescence intensities (TFI) greater than 150% of the DMSO control wells were flagged as fluorescent artifacts and excluded from further analysis. Next the remaining fragments were ranked according to percent inhibition of pKID binding across the three β-ME concentrations (Figure 2-3b). As expected, the highest stringency of 5 mM β-ME resulted in decreased inhibition relative to the lower β-ME concentrations. The two lower β-ME concentrations, 0.2 mM and 1 mM, deviated from this trend in which the lowest percent inhibition fluctuated between 1 mM β-ME and 0.2 mM β-ME for the different fragments.

From the top hits, 10 disulfides were cherry-picked for validation by LC-MS analysis (Figure 2-3c). All of these fragments covalently labeled the KIX domain. However 2E10 was likely a false positive given its high percent inhibition by FP yet a low degree of labeling by MS. Comparison of labeling across the three β-ME concentrations reveals the relative potencies of the fragments. From the ten hits, two fragments, 3D4 and 6D11 were chosen for further characterization. The fragment 3D4 labeled KIX H602C more effectively than any other fragments and maintained a high percent inhibition of pKID even at 5 mM β-ME (31%) (Figure 2-3d). The fragment 6D11 had the highest average percent inhibition of pKID for the three β-ME concentration. Notably, percent labeling did not always correlate with inhibition of pKID binding. While fragment 3D4 labelled KIX ~70% at 5 mM β-ME, this only resulted in 24% inhibition of pKID binding. The larger fragment 6D11 labelled KIX only ~15% at 5 mM β-ME, but this translated to nearly equivalent inhibition (22%) of pKID binding. This finding suggests that although 6D11 may exhibit weaker overall potency, it is the more efficient inhibitor. Other top hits replaced the diphenylamine moiety in 6D11 for a diphenylmethane or 2-chloro-2-N-phenylaniline. These changes did not improve their ability to displace the pKID tracer, but their ability to bind to the
KIX H602C mutant improved slightly.

For comparison to previous LC-MS Tethering screens, dose-response curves were determined for 3D4 and 6D11 using LC-MS to determine the percent of KIX H602C mutant bound to the fragment. (Figure 2-4a). The concentration at which 50% of the KIX H602C mutant is bound by the fragment (DR$_{50}$) was lower with the fragment 3D4 than 6D11. This finding was consistent with single point LC-MS measurements in which the fragment 3D4 was more potent than the fragment 6D11. The disulfide fragment library contained several variants of the core scaffolds of 3D4 and 6D11 and a comparison revealed that both structures are sensitive to substituent placement and identity; this is particularly true for 6D11 where modifications of the distal aromatic ring leads to dramatic changes in inhibition (Figure 2-S2).

For comparison to previous LC-MS tethering screens, dose-response curves were determined for 3D4 and 6D11 using LC-MS to determine the percent of KIX H602C mutant bound to the fragment (Figure 2-4a). The concentration at which 60% of the KIX H602C mutant is bound by the fragment (DR$_{50}$) was lower with the fragment 3D4 than 6D11. This finding was consistent with single point LC-MS measurements in which the fragment 3D4 was more potent than the fragment 6D11.

Finally, we labeled greater than 90% of KIX H602C with the 3D4 fragment and used this protein-ligand conjugate for direct binding experiments with the MLL, pKID, and c-Myb tracers. The equilibrium dissociation constant ($K_d$) of each tracer for the KIX-3D4 conjugate was assessed by a binding isotherm that accounts for ligand depletion.$^{10,21}$ Surprisingly, 3D4 not only increases the $K_d$ for binding to pKID (6.5-fold) and c-Myb (9-fold) but also MLL (9-fold), which binds on the opposite face of the KIX domain (Figure 2-4b). This finding demonstrates a negative allosteric coupling between the pKID- and MLL-binding sites, the first observation of this phenomenon. Given that 3D4 disrupts the binding of all three coactivator peptides, we are pursuing
irreversible covalent analogues as chemical probes for studying CBP/p300 biology.

In conclusion, FP Tethering is an efficient and accessible screening format for identifying fragments that disrupt a PPI. This method was successfully benchmarked against a previous MS-based Tethering screen on the MLL-binding site of KIX. Application of this method in discovery mode against a more challenging surface within KIX, the broad interface of the KIX:pKID complex, was also a success. FP Tethering lead to 9 fragments confirmed to displace pKID from the KIX domain and by covalently labeling the protein. These molecules represent promising leads for follow-up studies. More broadly, these results suggest that Tethering could be combined with any functional assay that is compatible with disulfide-containing molecules and β-ME, increasing the accessibility of this important technology.

Acknowledgements

T.J.R was supported by a predoctoral fellowship from the NIH (F31 CA180378). W.C.P. is thankful to the NIH for a postdoctoral fellowship (F32 GM090550). Aspects of this work were supported by NIH 2R01 GM65330 (to A.K.M.) and NIH R01AI070292 (J.W.) We thank Dr. Mark Burlingame for maintaining the Tethering library, Dr. Chris Wilson for assisting with automation equipment, and Drs. Olivier Julien and Zachary Hill for helpful discussions. We also thank Laura C. Cesa for providing several KIX cysteine mutant plasmids and Drs Ning-kun Wang and Chinmay Y. Majmudar for helpful discussions.
References


Figure 2-1. Tethering combined with a ligand displacement assay is used to identify chemical probes that disrupt the interaction between KIX and its binding partners. a. Comparison between traditional liquid chromatography-mass spectrometry (LC-MS)-based Tethering with the newly devised fluorescence polarization (FP)-based Tethering. b. Cartoon representation of the KIX domain (grey) bound to two coactivator peptides MLL (magenta) and pKID (cyan). (PDB IDs: 2AGH, 1SB0) Previously, four cysteines lining the MLL-binding pocket (left) were screened using the LC-MS-based Tethering. Here, we used FP-based Tethering to target the pKID-binding pocket by introducing a cysteine at position H602 (right).
Figure 2-2. FP- and MS-based Tethering identify overlapping ligand sets for the MLL-binding site. 

**a.** Dose response of previously identified small-molecule disulfides in a FP displacement assay with KIX N627C. 

**b.** The percent of MLL displaced from KIX N627C is shown for 70 small-molecule disulfides at both medium and high stringency (1 mM and 5 mM β-ME, respectively). The top 4 fragments from the previous MS-based Tethering are colored in red. 

**c.** The structures and properties of the top two fragments that were rediscovered by FP-based Tethering.
a

Screened 960 disulfides (100 μM) at low, medium, and high stringency (0.2 mM, 1 mM, and 5 mM β-ME)

Removed fluorescence artifacts
Identified 63 hits (>2σ)
6.6% hit rate

Selected 10 disulfides for validation by Mass Spectrometry
9 unique hits confirmed

b

Percent of pKID Displaced from KIX H602C

![Graph showing the percent of pKID displaced from KIX H602C with rank order on the x-axis and percentage on the y-axis.]

- 0.2 mM β-ME
- 1 mM β-ME
- 5 mM β-ME

![Graph showing the percent of KIX H602C labeled by compound.]

Percent of KIX H602C labeled by compound

<table>
<thead>
<tr>
<th>Compound Structure</th>
<th>Percent of pKID Displaced from KIX*</th>
<th>Percent of KIX labeled by compound*</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="3D4 structure" /></td>
<td>31%</td>
<td>71%</td>
</tr>
<tr>
<td><img src="image" alt="6D11 structure" /></td>
<td>22%</td>
<td>15%</td>
</tr>
</tbody>
</table>

*Tested at high stringency (5 mM β-ME)
Figure 2-3. FP-based Tethering screen identifies new ligands that disrupt the interaction between KIX and pKID. 

a. Overview of the workflow for screening, hit selection, and validation.

b. The percent of pKID displaced from KIX H602C is shown for 960 small-molecule disulfides at low, medium, and high stringency (0.2 mM, 1 mM, and 5 mM β-ME, respectively).

c. The percent of KIX H602C that is labeled by the top 10 small-molecule disulfides was measured by mass spectrometry. The reaction was performed with increasing stringency to permit assessment of relative affinities.

d. The structures and properties of ligands that displace pKID from KIX H602C.
Figure 2-4. Labeling of KIX H602C with 3D4 decreases affinity for both pKID and MLL.  

a. The dose-response binding experiment was performed with KIX H602C and fragments 3D4 and 6D11 using LC-MS analysis.  

b. Dose response of KIX H602C (closed circles) or KIX H602C labeled with 3D4 (open circles) against fixed concentration of fluorophore-tagged pKID, c-Myb, or MLL.
**Supplemental Material**

**Experimental Section**

**Expression and purification of the KIX domain**

The KIX domain (residues 586-672) of mouse CBP protein fused to a hexahistadine tag and short polar linker was cloned into the bacterial expression pRSETB vector. All cysteine mutants were generated by using site-direct mutagenesis as previously described.\(^1\,^2\) The KIX domain protein was overexpressed from Escherichia coli Rosetta2(DE3) pLysS (Novagen) cells in Terrific Broth (37° C, 250 rpm). When the cell density reached an OD600nm of ~0.8, protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for approximately 12 hours at 25° C. The cells were harvested by centrifugation and stored at -80° C. The hexahistadine-tagged KIX protein was affinity purified using Ni-NTA beads (Qiagen) followed by ion-exchange chromatography (Source S column, GE Healthcare) using an FPLC system. The purified protein was stored in 10 mM phosphate, 100 mM NaCl, pH 6.8 at -80°C.

**Synthesis of fluorescent peptides**

Peptides were synthesized using standard Fmoc solid phase synthesis as previously described.\(^3\)

**Fluorescent polarization (FP) binding assay**

In 384-well microplates varying concentrations of KIX domain protein were mixed in binding buffer (10 mM phosphate, 100 mM NaCl, pH 6.8) with a constant concentration of fluorescein isothiocyanate (FITC)-labeled peptides (25 nM) and β-mercaptoethanol (BME) to a final volume of 10 µL in each well. The samples were incubated at room temperature for 1 hour to reach equilibrium. The anisotropy and fluorescence intensity were monitored using the Tecan Genios Pro microplate reader at an excitation wavelength of 485 nm and an emission wavelength at
535 nm. The equilibrium dissociation constant (K_d) were calculated by fitting the observed FP or anisotropy values as a function of KIX protein concentration to the ligand depletion model assuming single site binding of peptide to protein,

\[
F = F_f + (F_b - F_f) \times \left( \frac{([L_T] + K_d + [P_T]) - \sqrt{([L_T] - K_d - [P_T])^2 - 4[L_T][P_T]}}{2[L_T]} \right),
\]

where \([L_T]\) and \([P_T]\) are the total concentrations of fluorescent peptide and KIX domain protein, respectively, \(F\) is the observed polarization, \(F_b\) is the maximum polarization value for the bound form of the fluorescent peptide, and \(F_f\) is the minimum polarization value for the free form of the fluorescent peptide. All nonlinear data analysis was performed with Prism 4 software (GraphPad Software).

**Fluorescence polarization Tethering screen**

KIX N627C (0.5 µM concentration) was mixed in binding buffer with FITC-labeled MLL peptide (25 nM), β-mercaptoethanol (1 mM or 5 mM), and one of 80 fragment disulfides (160 µM) in a final volume of 30 µL. For the screen with the KIX H602C mutant, the protein at a concentration of 4 µM was mixed in binding buffer with FITC-labeled pKID peptide (25 nM), β-mercaptoethanol (0.2 mM, 1 mM or 5 mM), and one of 960 fragment disulfides (125 µM) in a final volume of 20 µL. Additional information regarding the fragment library can be found in reference.\(^5\)

All plates were shaken at room temperature for 1h. The fluorescence polarization (FP) and total fluorescence intensity (TFI) were measured using the Analyst HT Multi-Plate Reader (Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength at 530 nm. Fragments that yielded TFI greater than 150% of the DMSO control were flagged as artifacts and excluded from further analysis. FP readings were transformed to percent inhibition, using the formula \([P_{Bound} - P_{Sample}] / [P_{Bound} - P_{Free}] \times 100\).
**Mass spectrometric validation of fragment hits**

KIX H602C (4 µM) was mixed in binding buffer with β-mercaptoethanol (0.2 mM, 1 mM or 5 mM) and each fragment disulfide (125 µM) in a total volume of 25 µL. The plate was shaken for 1 hour at room temperature to reach equilibrium. Each sample was subjected to LC-MS using an LCT Premier ESI-MS (Waters) with an inline C4 protein desalting column (Microtrap). Protein masses were deconvoluted using the Max-Ent algorithm within the MassLynx software. Percent labeling was measured by comparing the peak areas for the labeled versus unlabeled protein.

**Determination of dose response (DR50)**

A constant concentration of the KIX H602C mutant and fresh β-ME in 10 mM phosphate buffer (100 mM NaCl, pH 6.8) were incubated with varying concentrations of compound (0.2 µM -125 µM) at room temperature for one hour. The samples were centrifuged for 1 minute at 10,000 rpm and analyzed by mass spectrometry on an Agilent Q-TOF HPLC-MS instrument. Protein masses were deconvoluted using the Max-Ent algorithm within the Agilent MassHunter Workstation Bioconfirm software. The dose-response (DR_{50}) values were determined with a normalized response from 1 to 100 using the equation:

\[ y = \frac{100}{1 + 10^{(\log DR_{50}-x)}} \]

where \(x\) is the logarithm of the concentration of the fragment and \(y\) is the percent of the protein bound to the fragment.\(^4\)

**Fragment Tethering**

The KIX H602C mutant was incubated with 8 - 10 equivalents of small molecule and fresh β-ME in binding buffer (10 mM phosphate buffer, 100 mM NaCl, pH 6.8) and incubated overnight. Excess small molecule was removed and small molecule-protein complexes were concentrated
using 10 KD molecular weight cutoff concentrators (Vivascience). The extent of labeling was measured by Q-TOF LC-MS (Agilent). Protein complexes that were at least 90% alkylated were used for the FP binding assays.
Figure 2-S1. Validation of the FP Tethering competitive binding assay. A competition experiment was performed by using a constant concentration of KIX N627C mutant complexed with fluorescently-labeled MLL and varying the concentrations of the fragment 1A10, a known binder and inhibitor of the KIX domain and MLL.\textsuperscript{2,3} The polarization values were plotted against the varying concentrations of the fragment 1A10. Each data point represents an average of three independent experiments with the standard deviation. The IC\textsubscript{50} value was determined from the nonlinear regression equation for “log(inhibitor) vs response -- variable slope (four parameters)” using GraphPad Prism 4.00 software.
Figure 2-S2. Structure-activity relationships for compounds 3D4 and 6D11. A comparison of the inhibition values of 3D4, 6D11, and related fragments reveals that both scaffolds are sensitive to substituent placement and identity. Relative potency values are the inhibition value of each fragment measured under stringent (5 mM β-ME) conditions divided by the inhibition value for the parent fragment (3D4 or 6D11) and normalized to 1.
Table 2-S1. Mutation of His602 to Cys in KIX does not disrupt coactivator binding. The equilibrium dissociation constants ($K_d$) for the two tracers, pKID, and c-Myb were compared between the KIX H602C mutant and wild-type KIX using the FP direct binding assay described above. Here a constant concentration of tracer (25 nM) was mixed with increasing concentrations of the wild-type KIX domain or KIX H602C mutant (from 0 to 20 µM). The average anisotropy values from three independent experiments were used to determine the $K_d$ with the indicated error (standard deviation).

<table>
<thead>
<tr>
<th>$K_d$ (µM)</th>
<th>pKID</th>
<th>c-Myb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type KIX</td>
<td>0.48 ± 0.03</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>KIX H602C mutant</td>
<td>0.67 ± 0.06</td>
<td>1.8 ± 0.4</td>
</tr>
</tbody>
</table>
Supplemental References


2. N. Wang, C. Y. Majmudar, W. C. Pomerantz, J. K. Gagnon, J. D. Sadowsky, J. L.


4. J. D. Sadowsky, M. A. Burlingame, D. W. Wolan, C. L. McClendon, M. P. Jacobson and

Chapter 3

A small-molecule mimic of a peptide docking motif inhibits the kinase PDK1

T. Justin Rettenmaier, Jack D. Sadowsky, Nathan D. Thomsen, Steven C. Chen, Allison K. Doak, Michelle R. Arkin, and James A Wells
Abstract

There is great interest in developing selective protein kinase inhibitors by targeting allosteric sites, but these sites often involve protein-protein or protein-peptide interfaces that are very challenging to target with small molecules. Here, we present a systematic approach to target a functionally conserved allosteric site on the protein kinase PDK1 called the PIFtide binding site or PIF pocket. More than two dozen pro-survival and pro-growth kinases dock a conserved peptide tail into this binding site, which recruits them to PDK1 to become activated. Using a site-directed chemical screen, we identified and chemically optimized ligand-efficient, selective, and cell-penetrant small molecules (MW ~380 Da) that compete with the peptide docking motif for binding to PDK1. We solved the first high-resolution structure of a peptide docking motif (PIFtide) bound to PDK1 and mapped binding energy hot spots using mutational analysis. We then solved structures of PDK1 bound to the allosteric small molecules, which revealed a binding mode that remarkably mimics three of five hot-spot residues in PIFtide. These allosteric small molecules are substrate-selective PDK1 inhibitors when used as single agents, but when combined with an ATP-competitive inhibitor they completely suppress the activation of the downstream kinases. This work provides a promising new scaffold for the development of high-affinity PIF pocket ligands, which may be used to enhance the anticancer activity of existing PDK1 inhibitors. Moreover, our results provide further impetus to explore the helix aC patches of other protein kinases as potential therapeutic targets even though they involve protein-protein interfaces.
Introduction

Protein kinases are a rich source of targets for the development of chemical probes and therapeutics. However, the remarkable similarity of their ATP-binding pockets presents a formidable challenge for the development of selective ATP-competitive inhibitors. To address these limitations, efforts have focused on targeting allosteric sites in kinases. Exquisitely selective allosteric inhibitors of AKT, MEK, and ABL are now in clinical trials for cancer, and a number of other allosteric kinase inhibitors and activators are in preclinical development.\(^1\)

Despite these recent successes, finding allosteric modulators remains challenging because most allosteric opportunities are the sites of protein-protein or protein-peptide interactions, which are very difficult to mimic with small molecules. Moreover, traditional chemical screening approaches most often identify ligands for the more druggable ATP-binding pocket.

The helix αC patch is an ancient allosteric site that is present on a number of serine/threonine and tyrosine kinases.\(^2\) The binding of effector proteins to the helix αC patch activates some kinases and inhibits others. The helix αC patch is found most frequently in the AGC family of serine/threonine kinases, where this site is known specifically as the PDK1-interacting fragment (PIF) pocket. A hydrophobic motif (HM) found in the C-terminal tail of most AGC kinases must bind in cis to the PIF pocket for the kinase to be fully active. However, the AGC kinase PDK1 lacks its own HM and instead uses its PIF pocket as a docking site to recruit, phosphorylate, and thereby activate 23 other AGC kinases, including AKT, S6K, SGK, RSK, and PKC isoforms.\(^3\) The role of PDK1 as a master regulator of these pro-growth and pro-survival kinases has motivated the development of numerous PDK1 inhibitors as potential anticancer agents.\(^4\)

One strategy for inhibiting PDK1 has been to identify compounds that bind to its PIF pocket and disrupt the recruitment of substrates.

Early biochemical studies revealed that PIFtide, a synthetic peptide derived from the HM of PRK2, stimulates PDK1 activity towards a short peptide substrate\(^5\) but disrupts the recruitment
and phosphorylation of the full-length substrates S6K and SGK.\(^6\) Small-molecule mimics of PIFtide have been discovered through pharmacophore modeling\(^7\) and fragment-based approaches\(^8-10\), and some optimized analogs have been characterized structurally\(^10-13\). However, these compounds have limited membrane permeability, which diminishes their utility as chemical probes. Moreover, the lack of a structure of PIFtide bound to PDK1 has impeded the structure-based design of improved analogs that mimic the native allosteric interaction.

We have been exploring site-directed methods for targeting the PIF pocket of PDK1. Previously, we used a technique called disulfide-trapping (or Tethering) to discover small-molecule fragments (MW < 250 Da) that inhibited or activated PDK1 by covalently labeling a cysteine residue that was engineered into the PIF pocket.\(^10\) Here, we sought to discover non-covalent small molecules that could be used as chemical probes of PIF pocket function in cells. We developed a PIFtide competitive binding assay to perform a site-directed screen of \(~154,000\) compounds for new PIF pocket ligands. We discovered a novel series of diaryl sulfonamides (MW \(~380\) Da) that were chemically optimized and then characterized biochemically, structurally, and in cells. We also solved the first structure of PIFtide bound to PDK1, which reveals how small molecules mimic this peptide effector and provides new insights for the structure-based design of improved PIF pocket ligands. Remarkably, we found that PIF pocket ligands sensitize PDK1 to inhibition by an ATP-competitive inhibitor, enabling more complete suppression of downstream signaling in cells.

**Results**

**Site-directed chemical screen identifies diaryl sulfonamides as PIF pocket ligands**

To identify small molecules that bind to the PIF pocket of PDK1, we developed a fluorescence polarization (FP) competitive binding assay to screen for compounds that disrupt the interaction between PDK1 and PIFtide (**Figure 3-1a**). To achieve optimal sensitivity, FP competitive
binding assays require a fluorescent probe that binds tightly to the target \((K_d < 100 \text{ nM})\) and yields a large change in polarization signal when bound \((\Delta mP > 100 \text{ mP})\) (14). To develop a suitable PIFtide probe for the FP assay, we synthesized a panel of fluorescent PIFtides of varied length and amino acid content (full-length PIFtide: REPRILSEEQEMFRDFDYIADW). We found that removal of the first 13 amino acids, substitution of Phe14 and Phe17 with bromo-Phe residues, substitution of Tyr19 with Trp, and conjugation of 6-tetramethylrhodamine to the N-terminus resulted in a PIFtide probe with an ideal affinity and dynamic range \((K_d = 40 \text{ nM}; \Delta mP = 130 \text{ mP}; \text{Figure 3-S1})\). Tetramethylrhodamine, a red-shifted fluorophore relative to fluorescein, was selected to reduce the susceptibility of the assay to interference from autofluorescent compounds. The resulting competitive binding assay was suitably robust for a high-throughput screen \((Z' = 0.7)\).

The high-throughput screen workflow we employed is depicted in Figure 3-1b and is discussed in detail in the Supplementary Information. Briefly, we screened ~154,000 compounds at a single dose (33 µM) using the FP assay. We selected hits from this primary screen using a statistical threshold of 3σ (1460 hits; 0.9% hit rate). We filtered out autofluorescent artifacts by removing compounds that yielded total fluorescence intensities greater than 150% of the untreated control. We tested the remaining 1280 compounds using the FP assay in dose-response mode. To confirm hits in an orthogonal assay format, we selected the top 100 compounds for dose-response assessment in a surface plasmon resonance (SPR) assay that detects displacement of PDK1 from immobilized PIFtide. We confirmed the chemical identity of the top 15 hits by testing repurchased or resynthesized standards in the FP and SPR assays. One of the most potent hits was the diaryl sulfonamide RS-HTS (Figure 3-1c), which displayed a \(K_d\) of 15 µM in the FP assay and an \(IC_{50}\) of 20 µM in the SPR assay. Given the novelty of the diaryl sulfonamide scaffold, we decided to focus further efforts on optimizing and characterizing compounds in this class.
Iterative synthesis and characterization of improved sulfonamides

We next sought to improve upon the potency of RS-HTS via analog synthesis. We synthesized a panel of 300 diaryl sulfonamides and assessed the potency of each compound in the FP, SPR and kinase activity assays described above. These efforts yielded the regioisomers RS1 and RS2 as well as the inactive analog RSi (Figure 3-1c). RS1 and RS2 bound to PDK1 with a $K_d$ of 1.5 µM and 9 µM, respectively (Figure 3-1d). RS1 and RS2 stimulated the catalytic activity of PDK1 towards a peptide substrate by 2-fold and 6-fold, respectively (Figure 3-1e). By comparison, Δ8-PIFtide bound with a $K_d$ of 2 µM and stimulated PDK1 activity 4-fold, whereas the diaryl dicarboxylate PS210 bound with a $K_d$ of 3 µM and stimulated PDK1 activity 10-fold. In summary, RS1 and RS2 bound to PDK1 with an affinity similar to that of the 15-mer Δ8-PIFtide and the previously described PIF pocket ligand PS210, yet each molecule stimulated the catalytic activity of PDK1 to a different extent (2- to 10-fold).

It is well established that at high concentrations some small molecules form soluble aggregates, which assemble through a mechanism similar to micelles and can interact nonspecifically with proteins.16 We determined by dynamic light scattering that RS1 and RS2 were monomeric in solution at concentrations below 50 µM (Figure 3-S2a), which is more than 5-fold above their $K_d$ values. Moreover, the 6-fold stimulation of PDK1 activity by RS2 was not diminished in the presence of Triton X-100 or BSA (Figures 3-S2b,c), additives that are known to either disperse or mask soluble aggregates of small molecules.17 Thus, RS1 and RS2 do not modulate PDK1 activity through an aggregation-based mechanism.

While RS1 and RS2 stimulated PDK1 activity towards a short peptide substrate, we expected that these PIF pocket ligands would prevent PDK1 from phosphorylating most full-length protein substrates (e.g. S6K, SGK and RSK), because these substrates must dock into the PIF pocket of PDK1 to be efficiently phosphorylated in vitro6 and in cells18. Moreover, both a synthetic
PIFtide\(^6\) and a small-molecule mimic of PIFtide\(^7\) have been shown to inhibit the phosphorylation of S6K and SGK by PDK1 \textit{in vitro}. Accordingly, we found that 20 µM Δ8-PIFtide, RS1, or RS2 inhibited the \textit{in vitro} activation of S6K1 by PDK1 by 75%, 75%, and 60%, respectively (Figure 3-1f).

\textit{Structures of PDK1 bound to diaryl sulfonamides}

To reveal how RS1 and RS2 bind to PDK1, we determined the structures of these compounds in complex with PDK1. We first crystallized PDK1 in complex with ATP and then soaked these crystals with ligand to obtain the structures in complex with RS1 (1.6 Å resolution, Figure 3-2a) or RS2 (1.5 Å resolution, Figure 3-2b). Each structure showed unambiguous electron density for the bound compounds (Figures 3-S3a,b). The RS compounds share a binding mode in which the aromatic substituents bind to two adjacent subsites in the PIF pocket. The sulfonyl group of both RS compounds interacts with Arg131 through a salt bridge, since the sulfonamide is likely ionized under the crystallization conditions (pH 7.5; predicted pK\(_a\) ~6.5). To confirm the binding mode of the RS compounds, we attempted to disrupt compound binding by mutating Leu155, which resides in the back of PIF pocket and packs against both aromatic substituents. Mutation of Leu155 to Ala or Glu completely abolished the enhancement of PDK1 activity by Δ8-PIFtide, RS1, and RS2 (Figures 3-S4a-c). The Leu155Ala mutation also confers partial resistance to the RS compounds in the \textit{in vitro} S6K1 activation assay (Figure 3-S4d).

\textit{Molecular basis of PIFtide recognition by PDK1}

To reveal precisely how PDK1 recognizes a native peptide effector and to gain insight into the mimicry of this peptide by small molecules, we determined the crystal structure of a PDK1-PIFtide complex. We soaked crystals of PDK1 bound to ATP with PIFtides of varied length and obtained high quality diffraction data (1.4 Å resolution) with one variant, Δ8-PIFtide (residues 9-23). This structure showed unambiguous electron density for residues 13-21 of PIFtide (Figure
3-S3c) and reveals how PDK1 engages the core hydrophobic motif of PIFtide (MFxxFDYIA).

To accommodate PIFtide binding, the side chain of Arg131 swings out to open a hydrophobic channel and the side chain of Phe157 rotates \( \sim 90^\circ \) to make room for Phe17 of PIFtide. The conserved aromatic residues of PIFtide (Phe14, Phe17, and Tyr19) occupy three adjacent subsites within the PIF pocket (Figures 3-3a,b). Although the side chain of Asp16 could not be modeled, the Cα-Cβ bond vector for this residue appears to direct the side chain toward Arg131 on PDK1, suggestive of an electrostatic interaction. The conserved negatively-charged residue of PIFtide (Asp18) primarily interacts with Gln150, not Arg131 as was suggested previously.\(^1^9\) This charged binding mode is similar to that observed for the analogous Asp, phospho-Ser, or phospho-Thr in the HM of AKT\(^2^0\), S6K1\(^2^1\), and PKCβII\(^2^2\), respectively. Finally, Met13, Ile20, and Ala21 of PIFtide occupy small clefts at the periphery of the PIF pocket. A previously reported structure of an AKT chimera bound \textit{in cis} to PIFtide\(^2^0\) bears striking resemblance to the PDK1-PIFtide structure (PIFtide all atom RMSD = 1.6 Å; Figure 3-S5). In summary, PIFtide uses a three-pronged hydrophobic plug along with two anionic anchors to engage the PIF pocket of PDK1.

To determine the relative energetic contribution of each amino acid within the HM to binding, we individually mutated positions 10-19 of Δ8-PIFtide to alanine and measured the affinities of the mutant peptides using the FP competitive binding assay. Alanine scanning mutagenesis has been a reliable method for finding binding energy “hot spots” at protein-protein interfaces\(^2^3\), which often represent ideal small-molecule binding sites\(^2^4\). We found that the strongly conserved residues at positions 14, 17, 18, and 19 of PIFtide all constituted binding energy hot spots (\(\Delta\Delta G\) of 1.5-2.5 kcal/mol), whereas nonconserved residues 10-13 and 15 contribute little to binding affinity (\(\Delta\Delta G\) of 0-0.75 kcal/mol) (Figure 3-3c; each 1.4 kcal/mol increment in \(\Delta\Delta G\) is a 10-fold loss in affinity). Although Asp16 is not strongly conserved amongst HMs, mutation to Ala significantly affected binding affinity (\(\Delta\Delta G\) of 1.25 kcal/mol), further supporting an
electrostatic interaction with PDK1. These quantitative competitive binding data agree with qualitative immunoprecipitation binding data described previously. In summary, the HM of PIFtide contains five amino acids (FxDFDY) that constitute binding energy hot spots ($\Delta \Delta G > 1.25 \text{ kcal/mol}$).

**Mimicry of PIFtide by the RS compounds and PS210**

Comparing the binding modes of PIFtide and its small-molecule mimics, we find that side chains of Phe14 and Phe17 of PIFtide share a nearly identical trajectory with the aromatic substituents of the diaryl sulfonamides RS1 and RS2 (Figure 3-4a) and the diaryl carboxylate PS210 (Figure 3-4b). In addition, each compound class mimics one native electrostatic interaction: RS1 and RS2 mimic the interaction between Asp16 of PIFtide and Arg131 of PDK1, whereas PS210 mimics the interaction between Asp18 of PIFtide and Gln150 of PDK1. Neither class of compounds engages the hydrophobic pocket occupied by Tyr19/Ile20 of PIFtide. Overall, existing PIF pocket ligands mimic three of the five energetic hotspots of the PDK1-PIFtide interaction.

**RS1 binds to PDK1 selectively**

To determine whether RS1 binds to PDK1 selectively, we tested the effect of 10 µM RS1 on the catalytic activity of 39 of the 60 AGC family kinases, because these kinases all bind to a HM in their respective PIF pockets. We also tested IGF-1 receptor and mTOR, which directly impact the PDK1 signaling pathway that we intended to study, as well as the Aurora kinases. The strongest hits from this independent screen were a 90% stimulation of PDK1 activity and a 33% inhibition of MSK2 activity (Figure 3-S6). At 10 µM, RS1 did not inhibit 41 of the 44 kinases by more than 20%, which suggests that RS1 binds to PDK1 selectively.
Sulfonamides prevent S6K1 activation but permit AKT activation in cells

Having established that RS1 binds selectively to the PIF pocket of PDK1, we wanted to determine its effects on PDK1 signaling in cells. For these cell-based experiments, we used the inactive analog RSi to control for nonspecific effects of RS1, the highly selective ATP-competitive PDK1 inhibitor GSK2334470 (GSK)\textsuperscript{26,27} as a control for pathway modulation, and the diaryl dicarboxylate PS210 and its diester prodrug PS423 as a chemically distinct class of PIF pocket ligands.\textsuperscript{13}

We first tested whether RS1 would inhibit S6K1 activation in cells, as we found \textit{in vitro}. We serum-starved HEK293 cells, treated them with increasing concentrations of RS1 or control compounds, and then stimulated cells with IGF-1 for 15 minutes prior to lysis. To observe the activation state of S6K1 in cells, we monitored phosphorylation of its substrate ribosomal protein S6 by quantitative immunoblot using infrared dyes. Treatment with increasing doses of RS1 led to a dose-dependent but incomplete blockade of S6 phosphorylation (\textit{Figure 3-5a}). At 30 µM, S6 phosphorylation was inhibited by 50% by RS1 and 70% by PS423. Importantly, the inactive analog RSi had no effect on S6 phosphorylation, suggesting that RS1 must specifically bind to PDK1 to exert its effect. The diaryl dicarboxylate PS210 had no effect on substrate phosphorylation at 100 µM, confirming that its carboxylate groups must be masked as esters to be cell active. Thus, both RS1 and PS423 prevent the activation of S6K1 in cells, although this effect did not saturate at the doses tested.

We next assessed the effect of RS1 on the activation of AKT, which does not require binding of its HM to the PIF pocket of PDK1 to be efficiently activated.\textsuperscript{6,18} We treated cells the same as described for monitoring S6K1 activation but instead monitored the phosphorylation of AKT at Thr308 by PDK1. Treatment with increasing doses of RS1 exerted little effect on the phosphorylation of AKT (\textit{Figure 3-5b}). At 30 µM, AKT phosphorylation was inhibited by 10% by
**RS1** and 20% by **PS423**. At 10 µM, **GSK** inhibited AKT phosphorylation by only 50%, which is consistent with previous reports.\textsuperscript{27,28} Control compounds **RSi** and **PS210** did not affect AKT activation. In short, PIF pocket ligands largely permit the activation of AKT by PDK1.

**PIF pocket ligands enhance the ability of an ATP-competitive inhibitor to block PDK1 signaling**

Previous characterization of GSK2334470 (**GSK**) in cells revealed that the activation of AKT by PDK1 is much less sensitive to inhibition by this ATP-competitive inhibitor than is the activation of S6K, SGK, or RSK.\textsuperscript{27} The insensitivity of AKT activation is not due to alternative pathways for AKT activation since PDK1-deficient cells are incapable of activating AKT.\textsuperscript{18} Disrupting the capacity of AKT to bind to either PIP\textsubscript{3} or to the PIF pocket of PDK1 markedly sensitized AKT phosphorylation to inhibition by **GSK**, suggesting that having multiple recruitment mechanisms contributes to insensitivity to **GSK**.\textsuperscript{28} Given these data, we wondered whether **RS1** would enhance the ability of **GSK** to block the activation of AKT by disrupting the PIF-pocket-dependent recruitment of substrates to PDK1.

To test whether **RS1** enhances the efficacy of **GSK**, we serum-starved HEK293 cells, treated them with increasing doses of **GSK** with or without 30 µM **RS1**, and then stimulated the cells for 15 minutes with IGF1 prior to lysis. We monitored both S6K1 activation and AKT activation by immunoblot, as described above. Phosphorylation of S6 was much more sensitive to inhibition by **GSK** than was AKT phosphorylation, consistent with previous reports (Figures 3-6a,b).\textsuperscript{27,28} Co-treatment with **RS1** and **GSK** resulted in enhanced inhibition of S6 phosphorylation, which reflects additivity of each compound’s effect (Figures 3-6a and 3-S7). However, even though **RS1** did not block AKT activation by itself (Figure 3-5b), **RS1** enhanced the ability of **GSK** to block AKT phosphorylation (Figure 3-6b). Thus, combining **RS1** with **GSK** more effectively blocks the activation of both S6K1 and AKT in cells.
Discussion

Using a site-directed chemical screen, we have discovered a new class of diaryl sulfonamide compounds that bind to the PIF pocket of PDK1 and disrupt its signaling in cells. Key to our success was the development of a FP competitive binding assay, which we expect could be readily adapted to target the PIF pockets other AGC kinases. Our structures of PDK1 bound to PIFtide or its small-molecule mimics provide new insights for the structure-based design of PIF pocket ligands with improved affinity. Unlike previously reported PIF pocket ligands, the diaryl sulfonamide compounds freely diffuse into cells. These compounds are substrate-selective inhibitors of PDK1 as single agents, but in combination with an ATP-competitive inhibitor they completely suppress the activation of downstream kinases in cells.

We were surprised that both RS1 and PS210 bound to PDK1 with an affinity comparable to that of the 15-mer peptide Δ8-PIFtide (Figure 3-1d). Alanine scanning mutagenesis revealed that five of the six residues within the HM of Δ8-PIFtide are critical for binding (Figure 3-3c), yet RS1 and PS210 each lack a native electrostatic interaction (either Asp18 or Asp16, respectively) and a hydrophobic interaction (Tyr19). Thus, RS1 and PS210 make much more efficient contacts with the PIF pocket than PIFtide does (Ligand Efficiency: 0.35, 0.29, and 0.08 kcal/mol per heavy atom, respectively). Synthesizing analogs of RS1 or PS210 that mimic the native interactions they lack could improve their affinity for PDK1.

The diaryl sulfonamide RS1 and the diaryl dicarboxylate PS210 bind to PDK1 with similar affinity and both compounds appear to bind to PDK1 selectively. However, the sulfonamides hold a significant advantage over existing carboxylates: RS1 freely diffuses into cells whereas PS210 does not. This disparity in cellular permeability may be attributable to the difference between the pKₐ of the carboxylate and N-arylsulfonamide moieties. At physiological pH, only a negligible fraction of carboxylate is protonated and therefore cell-permeable (pKₐ ~3), whereas a
significant portion of the sulfonamide is protonated (pK\textsubscript{a} ~6.5). The carboxylates of PS210 can be masked as esters to create the cell-permeable prodrug PS423, which is hydrolyzed by intracellular esterases and accumulates within cells.\textsuperscript{29} However, the purpose of an ester prodrug is to improve oral bioavailability of a drug by improving absorption in the gut.\textsuperscript{30} Following entrance into circulation, PS423 would likely be rapidly hydrolyzed by esterases in the blood and liver,\textsuperscript{31} which would limit the delivery of active (cell-permeable) drug to target tissues. In summary, the diaryl sulfonamides represent a promising new chemical scaffold for the development of high-affinity PIF pocket ligands that freely diffuse into cells.

Since PDK1 is an essential mediator of PI3K-AKT growth signaling, many PDK1 inhibitors have been developed as potential anticancer therapies.\textsuperscript{3} However, two major findings diminished optimism for PDK1 as an oncology target. First, sustained knockdown of PDK1 levels by ~90% failed to prevent AKT activation or block tumor formation in PTEN-null mice.\textsuperscript{32} Second, the first potent and highly selective PDK1 inhibitor to be extensively characterized, GSK2334470 (GSK), did not significantly impact tumor growth in xenograft models.\textsuperscript{26} The failure of PDK1 inhibitors as anticancer agents may reside in their inability to effectively block the activation of AKT, even at concentrations that should inhibit >90% of PDK1 within a cell. Here, we show that a small molecule that disrupts the recruitment of AKT by the PIF pocket of PDK1 induces sensitivity to an ATP-competitive inhibitor (Figure 3-6b). The efficacy of this drug combination is not likely due to RS1 increasing the affinity of PDK1 for the active-site inhibitor GSK, since binding at the ATP-binding pocket and the PIF pocket were not cooperative \textit{in vitro} (Figure 3-S8). Rather, the ability of the PIF-pocket ligand (RS1) to interfere with PDK1’s capacity to recruit substrates likely lowers the threshold of active-site occupancy needed to effectively block PDK1 signaling with an ATP-competitive inhibitor (GSK).

Taken together, our findings pave the way for the development of potent PIF pocket ligands that freely diffuse into cells. Next-generation analogs can be combined with ATP-competitive
inhibitors to determine whether the complete suppression of PDK1 signaling observed here yields favorable outcomes in cancer models. Moreover, such a dual-targeting approach may also overcome the emergence of drug resistance, as has been demonstrated previously for targeting Bcr-Abl in CML.\textsuperscript{33} Broadly, the site-directed approach described here could be adapted to target the helix αC patch of other protein kinases, which may facilitate the identification of ligands that mimic these challenging protein-protein and protein-peptide interactions.

**Materials and Methods**

**Protein Expression and Purification.** PDK\textsubscript{150-359} (wild-type, Y288G Q292A, L155A, or L155E) and S6K\textsubscript{124-421} T412E were expressed in Sf9 insect cells using the Bac-to-Bac system (Invitrogen). Proteins were purified by Ni-NTA affinity chromatography and size-exclusion chromatography (details are provided in the Supplemental Material).

**High-Throughput Screen.** The overall screening strategy is depicted generally in Figure 3-1b (details are provided in the Supplemental Material).

**Chemical Synthesis.** Detailed methods for the synthesis and characterization of the RS compounds, PS210, PS423, and the peptides are provided in the Supplemental Material.

**Protein Kinase Activity Assays.** The catalytic activity of PDK1 towards the peptide substrate T308tide\textsuperscript{10} or the protein substrate S6K\textsubscript{1}\textsuperscript{6} were measured using radioactivity-based kinase assays as described previously.

**Crystallization, Data Collection, and Refinement of PDK1 complexes.** Crystals were obtained using a PDK\textsubscript{150-359} mutant (Y288G Q292A) that disrupts a crystal contact that normally prevents ligands from binding to the PIF pocket.\textsuperscript{11} PDK1 was crystallized in complex with ATP and crystals were soaked with ligand overnight prior to harvesting. Diffraction data were collected at ALS Beamline 8.3.1. More details on the crystallization conditions and structure
solution are provided in the Supplemental Material. Final refinement statistics are summarized in Table 3-S1.

**Cell culture studies.** HEK293 cells were cultured in DMEM high glucose medium supplemented with sodium pyruvate, nonessential amino acids, penicillin/streptomycin, and 10% FBS. Cells were serum starved for 16 h prior to drug treatment for 1 h, stimulation with IGF1 for 15 min, and lysis (more details are provided in the Supplemental Material).

**Statistics.** All statistical analyses were performed using GraphPad Prism 6. All scatterplot bars and bar graphs depict means of data. All error bars are ±SD. Statistical significance was calculated using an unpaired two-sided t-test assuming equal SD. The IC$_{50}$ and EC$_{50}$ values were calculated using a sigmoidal dose-response equation with variable slope. IC$_{50}$ values from the FP competitive binding assay were converted to $K_d$ values using an equation that corrects for ligand depletion.$^{34}$

**Acknowledgements**

We thank members of the Wells lab for helpful suggestions and critical review of the manuscript. We also thank the staff of ALS Beamline 8.3.1. This work was supported by a NIH grant (R01 CA136779 to J.A.W.), a NIH predoctoral fellowship (F31 CA180378) and Krevans fellowship to T.J.R., and postdoctoral fellowships from the California Tobacco Related Disease Research Program (110385 to J.D.S) and the Damon Runyon Cancer Research Foundation (2082-11 to N.D.T).
References


Figure 3-1. Discovery and optimization of diaryl sulfonamides as PIFtide mimics.

a. Schematic of a FP competitive binding assay developed to identify small-molecule mimics of the PIFtide. b. Overview of the high-throughput screen and triage process. c. Chemical optimization of the diaryl sulfonamide hit from HTS. RSi is an inactive analog used as a negative control. PS210 is a known PIF pocket ligand used as a positive control. d. Dose-response curves for PIFtide, the RS compounds, and PS210 in the FP competitive binding assay. e. Dose-response curves for PIFtide and the RS compounds in a radioactive kinase activity assay that monitors the phosphorylation of T308tide peptide substrate by PDK1. f. Effect of PIFtide and the RS compounds on the in vitro activation of S6K1 by PDK1. Following activation of S6K1 by PDK1 for 30 min, the kinase activity of S6K1 was determined by a radioactive kinase assay using the Crosstide substrate. The activity of S6K1 alone was used for normalization (dotted line). Error bars are ±SD (n=3).
Figure 3-2. Structures of the RS compounds bound to the PIF pocket of PDK1. a. Structure of the PDK1- RS1 complex. PDK1 is shown as a yellow surface and both ATP and RS1 are shown as white sticks colored by heteroatom. The relative orientation of the ATP-binding site and the PIF pocket is depicted. b. Close-up view of the PDK1-RS1 interaction. c. Close-up view of the PDK1-RS2 interaction.
Figure 3-3. Structural and energetic analysis of the PDK1-PIFtide interaction. a. Structure of the PDK1-PIFtide complex. PDK1 is colored yellow and PIFtide is shown as purple sticks. b. Sequence logo depicting the consensus hydrophobic motif from 28 AGC kinases that are known or inferred to interact with PDK1. The consensus sequence is xFxxF[-](Y/F)(V/A/I)x, where [-] indicates a negatively charged residue (Asp, Glu, or phosphorylated Ser/Thr). c. Hot spot analysis of the PDK1-PIFtide interaction. Residues 10-19 of PIFtide9-23 were subjected to alanine scanning. The residues colored red constitute the hydrophobic motif. Energetic contributions were determined from the $K_i$ values for mutant peptides using the formula $\Delta \Delta G = 1.4\text{(kcal/mol)} \times \log(K_{i,\text{mut}}/K_{i,\text{wt}})$. 
Figure 3-4. Mimicry of PIFtide by diaryl sulfonamide and diaryl dicarboxylate compounds.

a. Overlay of the binding modes of PIFtide and the RS compounds. This view highlights the similar trajectory of the FxxF motif of PIFtide and the aromatic substituents of the RS compounds. b. Overlay of the binding modes of PIFtide and the diaryl dicarboxylate PS210 (PDB ID: 4AW1).
**Figure 3-5. PIF pocket ligands block S6K1 activation but only weakly affect AKT**

**activation in cells. a.** Effect of increasing doses of RS1 or control compounds on the phosphorylation of S6. Cell lysates were immunoblotted with antibodies for phospho-S6 S235/6 and α-tubulin. **b.** Effect of increasing doses of RS1 or control compounds on the phosphorylation of AKT. Same experiment as in a, except antibodies against phospho-AKT T308 and α-tubulin were used. The level of phosphorylation was quantified from infrared signal and normalized to the α-tubulin signal. Error bars are ±SD (n=2). Drugs used: GSK is a selective ATP-competitive inhibitor of PDK1. PS210 is a dicarboxylate PIF pocket ligand that does not enter cells. PS423 is a diester prodrug of PS210.
Figure 3-6. RS1 enhances the effect of a PDK1 active-site inhibitor to more effectively block both S6K1 and AKT activation. a. Effect of RS1 on the dose-dependent inhibition of S6 phosphorylation by the PDK1 inhibitor GSK2334470. Cell lysates were immunoblotted with antibodies for phospho-S6 S235/6 and α-tubulin. b. Effect of RS1 on the dose-dependent inhibition of AKT phosphorylation by the PDK1 inhibitor GSK2334470. Same experiment as in a, except cells were immunoblotted with antibodies for phospho-AKT T308 and α-tubulin. The level of phosphorylation was quantified from infrared signal and normalized to the α-tubulin signal. Scatterplots represent data pooled from 5 independent experiments. Statistical significance was calculated using an unpaired two-sided t-test (*P < 0.05, **P < 0.01, ***P < 0.001).
Supplemental Material

**Experimental Section**

**Reagents and Antibody Sources**

GSK2334470 (>98% pure) was purchased from Sigma and used without further purification. Human IGF1 (#8917SC) and antibodies to phospho-S6 S235/6 (#4858) (1:2000) and phospho-AKT T308 (#13038) (1:1000) were purchased from Cell Signaling. The antibody to α-tubulin (T6199) (1:5000) was purchased from Sigma. The IR dye secondary antibodies Goat anti-Mouse 680RD (1:10000) and Donkey anti-Rabbit 800CW (1:10000) were purchased from Li-COR Biosciences.

**Protein Expression and Purification**

PDK1<sub>50-359</sub> (wild-type, Y288G Q292A, L155A, or L155E) and S6K1<sub>24-421 T412E</sub> were expressed in Sf9 insect cells using the Invitrogen Bac-to-Bac protocol. Each kinase was cloned into the pFastBac HTB vector with an N-terminal 6xHis tag followed by a TEV protease cleavage site. During log-phase growth (~2x10<sup>8</sup> cells/mL), Sf9 cells were infected with baculovirus (MOI: 2) and grown for 72 hr. Cell pellets were lysed with a detergent-based Lysis buffer for 30 min on ice with intermittent swirling. Cell lysates were clarified by centrifugation and sterile filtration. Following purification by Ni-NTA affinity chromatography (GE Healthcare), 6xHis-TEV protease (1:40 w/w) was added and the sample was dialyzed overnight into Gel filtration buffer. Next, the sample was spiked with 20 mM imidazole and passed through a Ni-NTA column. The cleaved kinase without its 6xHis tag was collected in the flow-through at >95% purity. Finally, the sample was concentrated to ~2 mL and run on a HiLoad 16/60 Superdex 200 (GE Healthcare) gel filtration column to isolate non-aggregated kinase. Protein identity and phosphorylation state was assessed by LC-MS of the intact protein using an LCT Premier mass spectrometer (Waters). All purified proteins were concentrated with ultrafiltration centrifugal filters (Millipore),
snap frozen in liquid nitrogen, and stored at -80°C.

*Lysis buffer:* 25 mM Tris, pH 7.5, 150 mM NaCl, 20 mM imidazole, 5 mM NaF, 1 mM Na$_3$VO$_4$ (activated), 1 mM MgCl$_2$, 5% v/v glycerol, and 0.5% v/v Igepal CA-630. Just before lysis, add 100 µg/mL DNAse I, 100 µg/mL RNAse A, and an EDTA-free protease inhibitor tablet (Roche).

*Gel filtration buffer:* 25 mM Tris, pH 7.5, 300 mM NaCl, 5% v/v glycerol, 2 mM DTT

**High-Throughput Screen**

The screening library consisted of 153,888 compounds purchased from ChemDiv, Chembridge, and Microsource Discovery Systems. The primary screen was conducted at a single dose (33 µM) using the FP competitive binding assay (*Figure 3-1a*) in a 15 µL volume in 384-well black plates (Corning #3676). PDK1 (200 nM) and the fluorescent PIFtide probe (50 nM) were combined in a buffer containing 25 mM Tris, pH 7.5, 125 mM NaCl, and 0.0625% v/v Pluronic F-68. Next, 50 nL of compound in DMSO was pin-transferred into each well using a BioMEK FX (Beckman Coulter). Following incubation for 1 hr, the fluorescence polarization value was read using the Analyst HT plate reader (LJL Biosystems; Ex: 530 nm; Em: 580 nm). Wells without PDK1 were used as positive controls and wells treated with DMSO were used as negative controls. This assay showed robust performance during the primary screen with an average Z’ factor of 0.7.

Hits from the primary screen were selected using a statistical threshold of 3σ (1460 hits, 0.9% hit rate). Compounds that yielded a total fluorescence intensity (TFI) greater than 150% of the mean of the DMSO control wells were flagged as autofluorescent artifacts and excluded from further analysis. The remaining 1280 compounds were subjected to an 8-point dose response in the FP competitive binding assay. Based on potency and hill slope ($n < 2$), 100 hits were
selected for validation by dose-response in a SPR assay that monitors displacement of PDK1 from immobilized PIFtide using a Biacore T100 (GE Healthcare). Finally, the top 10 hits were repurchased or resynthesized and retested in the FP and SPR assays to confirm their chemical identity.

Crystallization of PDK1 and Soaking with Ligands
All crystals were obtained using a PDK1<sub>50-359</sub> double mutant (PDK1dm; Y288G Q292A), which was designed to disrupt a crystal contact that normally prevents ligands from binding to the PIF pocket. The two mutated residues are located in the αG helix in the C-terminal lobe of the kinase, which is more than 30 Å away from the PIF pocket. PDK1dm was concentrated to 28 mg/mL in a buffer containing 25 mM Tris, pH 7.5, 500 mM NaCl, and 1 mM DTT. Next, EDTA (16.6 mM) and ATP (9 mM, pH 7) were added resulting in a final protein concentration of 21 mg/mL. Hanging drops were set using a Mosquito Crystal (TTP Labtech) with 100 nL protein and 100 nL precipitant solution (0.1 M HEPES, pH 7.5, 1.2M sodium citrate) per drop. Crystals appeared within 3 days and were soaked with drug on day 5. Crystal-soaking solutions were made by adding drug to 1 mM in a mother liquor containing 90% of the contents of the dehydrated crystal drops (22.5 mM Tris pH 7.5, 450 mM NaCl, 0.9 mM DTT, 0.09 M HEPES, pH 7.5, 1.1M sodium citrate, 8 mM ATP, 15 mM EDTA). Crystal-soaking solution (1 µL) was added to each drop and allowed to soak overnight. Individual crystals were looped and transferred into a cryoprotectant solution (crystal-soaking solution with 25% v/v glycerol) to equilibrate for 5 min and were then flash frozen in liquid nitrogen.

Structure Solution and Refinement
Diffraction data was indexed and scaled using HKL-2000. Structures were solved using molecular replacement with the structure of PDK1 bound to PS210 (PDB ID 4AW1) as a search model in Phaser. Iterative model building and refinement was performed with Coot and
PHENIX\textsuperscript{6}, respectively. Structure validation was performed using MOLProbity\textsuperscript{7}.

\textit{Consensus Sequence for HM}

The consensus hydrophobic motif (HM) sequence was determined from 27 kinases that are known or inferred to interact with PDK1 (AKT1,2,3; PKA\textsubscript{α,β,γ}; PKC\textsubscript{α,β,γ,δ,ε,θ,η,ι,ζ}; PKN1,2,3; ROCK1; RSK1,2,3,4; S6K1,2; SGK1,2,3). Reference protein sequences were derived from Uniprot. Sequence logos were created using WebLogo 3.4.\textsuperscript{8}

\textit{Monitoring the Activation of AKT and S6K1 in Cells}

All drug-treatment experiments were conducted in 12-well tissue culture plates that were pre-treated for 1 hr at 37°C with 50 μg/mL Poly-D-Lysine (MW 70K-150K; Sigma) dissolved in sterile water. HEK293 cells (200,000) were seeded into each well a 12-well plate and allowed to grow in complete media for 24 hours. The cells were then serum-starved overnight (16 hr) prior to drug treatment. All drug solutions were made at 1X concentration in serum-free media from concentrated DMSO stocks and the final DMSO concentration was fixed at 0.2%. Following serum-starvation, cells were exchanged into 1 mL of serum-free media with 1X drug. After 1 hr of drug treatment, cells were stimulated with 50 ng/mL IGF1 by the addition of 1 mL of serum-free media containing 1X drug and 2X IGF1. After 15 min of stimulation, media was aspirated, cells were washed once with ice-cold PBS, and then cells were lysed by the addition of 100 μL of M-PER lysis buffer (Thermo Scientific) containing protease inhibitor cocktail (Sigma), Tyr and Ser/Thr phosphatase inhibitor cocktails (Sigma), 2 mM Na\textsubscript{3}VO\textsubscript{4} (activated), 1 mM PMSF, and 10 μM GSK2334470. Cells were scraped with a rubber policeman, lysates were transferred to microcentrifuge tubes, and cellular debris was pelleted by spinning at 20K x g on a tabletop centrifuge for 15 min at 4°C. The clarified lysates were subsequently separated by SDS-PAGE and analyzed by IR Western Blot using an Odyssey Classic Imager (Li-COR Biosciences). All blots were scanned using the membrane present with a 700 Laser Intensity of 2.0 and an 800
Laser Intensity of 5.0. Western Blot band intensities were quantified using the ImageStudioLite package (Li-COR Biosciences) using left/right median background subtraction. The phospho-protein signal in each lane was normalized by the signal of the loading control α-tubulin. Next, the ratio of phospho-protein/α-tubulin for DMSO control lane was normalized to 1.0.

General Methods for Chemical Synthesis

All air or moisture sensitive reactions were performed under argon in oven-dried glassware. Chemical reagents and anhydrous solvents were obtained from commercial sources and used as-is. Flash chromatography purification was performed using a Biotage Isolera with prepacked silica columns (Silicycle). Reverse phase purification was performed on a Waters semi-preparative HPLC with C18 Xterra column (Waters). The mobile phase consisted of methanol and water (each containing 0.2% formic acid). LCMS data was acquired on a Waters 2795 Analytical HPLC equipped with a photodiode array detector, evaporative light scattering detector, and ZQ MS detector. LCMS analysis was performed using a gradient of 5-95% methanol in water (each containing 0.2% formic acid) over 8 minutes. \(^1\)H and \(^{13}\)C NMR data were collected on a Varian 400 MHz spectrometer in \(d_6\)-DMSO. Chemical shifts are reported relative to TMS. All of the small molecules used in this study were found to be greater than 95% pure based on LCMS and NMR analysis.

All peptides were synthesized using Fmoc chemistry on Rink Amide AM resin (EMD Biosciences) following standard procedures. Peptides were cleaved from the resin using a cocktail of trifluoroacetic acid, triisopropylsilane, and water (95:2.5:2.5) and precipitated in ice-cold ether. Peptides were purified by RP-HPLC using a C18 Xterra column (Waters) and then lyophilized. The identity of each peptide was confirmed by LC-MS and peptide purity exceeded 90% in all cases.
RS1 and RS2 were synthesized by combining 1 equivalent amine (0.2 M) and 1.5 equivalent sulfonyl chlorides in pyridine and heating to 95°C for 4 h. The pyridine was evaporated and then azeotroped with n-heptanes. The crude reaction mixture was dissolved in DMSO and purified by RP-HPLC. Fractions were analyzed by LC-MS, combined, and then lyophilized to yield to final products. Yields typically exceeded 80%. PS210 and the diester PS423 were synthesized and purified as described previously.¹⁰

N-(6-chlorobenzo[d]thiazol-2-yl)benzo[b]thiophene-3-sulfonamide (RS1):

![RS1 structure](image)

¹H NMR (400 MHz, d₆-DMSO): δ 13.44 (broad s, 1H), 8.54 (s, 1H), 8.14 (d, J=6.4 Hz, 1H), 8.10 (d, J = 6.4 Hz, 1H), 7.98 (d, J = 1.6 Hz, 1H), 7.53 (dt, J = 6.4, 0.8 Hz, 1H), 7.48 (td, J = 6.4, 0.8 Hz, 1H), 7.42 (dd, J = 7.2, 1.6 Hz, 1H), 7.27 (d, J = 6.4 Hz, 1H); ¹³C NMR (125 MHz, d₆-DMSO): δ 167.2, 140.0, 135.4, 134.8, 133.7, 133.6, 127.7, 127.4, 126.8, 125.6, 125.5, 123.4, 123.2, 122.5, 114.3; LCMS (m/z): [M+H]^+ calcd., 380.96; found, 381.0

N-(6-chlorobenzo[d]thiazol-2-yl)benzo[b]thiophene-2-sulfonamide (RS2):

![RS2 structure](image)

¹H NMR (400 MHz, d₆-DMSO): δ 8.05-8.0 (m, 4H), 7.49 (td, J = 7.2, 1.6 Hz, 2H), 7.44 (dd, J = 8.8, 2.4 Hz, 1H), 7.33 (d, J = 8.4 Hz, 1H); ¹³C NMR (125 MHz, d₆-DMSO): δ 168.0, 140.5, 137.4, 127.5, 127.4, 127.1, 125.7, 125.5, 123.0, 122.4, 114.7; LCMS (m/z): [M+H]^+ calcd., 380.96; found, 381.0
2-(3-oxo-1-phenyl-3-(4-(trifluoromethyl)phenyl)propyl)malonic acid (PS210):

\[
\begin{align*}
\text{H NMR (400 MHz, } d_6-\text{DMSO): } & \delta 8.04 (d, J = 8.0 \text{ Hz, } 2\text{H}), 7.86 (d, J = 7.6 \text{ Hz, } 2\text{H}), 7.27 (d, J = 7.2 \text{ Hz, } 2\text{H}), 7.20 (t, J = 7.6 \text{ Hz, } 2\text{H}), 7.12 (t, J = 7.2 \text{ Hz, } 1\text{H}), 3.87 (td, J = 10.8 \text{ Hz, 3.6 Hz, } 1\text{H}), 3.74 (d, J = 10.8 \text{ Hz, } 1\text{H}), 3.60 (dd, J = 17.2 \text{ Hz, 9.6 Hz, } 1\text{H}), 3.42 (d, J = 17.2 \text{ Hz, } 1\text{H}). \text{ LCMS (m/z): } [\text{M+H}]^+ \text{ calcd., 380.09; found, 380.1}
\end{align*}
\]

bis(acetoxyethyl) 2-(3-oxo-1-phenyl-3-(4-(trifluoromethyl)phenyl)propyl)malonate (PS423):

\[
\begin{align*}
\text{H NMR (400 MHz, } d_6-\text{DMSO): } & \delta 8.03 (d, J = 10.0 \text{ Hz, } 2\text{H}), 7.88 (d, J = 10.4 \text{ Hz, } 2\text{H}), 7.28-7.34 (m, 2\text{H}), 7.23 (t, J = 9.2 \text{ Hz, } 2\text{H}), 7.16 (t, J = 9.6 \text{ Hz, } 1\text{H}), 5.72 (dd, J = 14.4, 6.0 \text{ Hz, } 2\text{H}), 5.45 (q, J = 5.2 \text{ Hz, } 2\text{H}), 4.19 (d, J = 10.4 \text{ Hz, } 1\text{H}), 3.94 (td, J = 9.2, 4.0 \text{ Hz, } 1\text{H}), 3.73 (dd, J = 17.6, 9.6 \text{ Hz, } 1\text{H}), 3.44 (dd, J = 17.6, 4.0 \text{ Hz, } 1\text{H}), 2.05 (s, 3\text{H}), 1.93 (s, 3\text{H}). \text{ LCMS (m/z): } [\text{M+H}]^+ \text{ calcd., 525.13; found, 525.1}
\end{align*}
\]
Figure 3-S1. Optimized FP probe binds to PDK1 with high affinity and yields a large dynamic range. Increasing concentrations of PDK1 were incubated with 50 nM of the optimized FP probe until equilibrium binding was achieved. Error bars are ±SD (n = 2). Probe sequence: 6-TAMRA-(2-bromo-Phe)-Arg-Asp-(3-bromo-Phe)-Asp-Trp-Ile-Ala-Asp-Trp-CONH₂.
Figure 3-S2. Diaryl sulfonamides modulate PDK1 through a specific interaction. a. Dynamic light scattering measurements of increasing concentrations of RS1 or RS2 in PDK1 kinase activity assay buffer. DMSO in buffer is used as a control. Both RS1 and RS2 aggregated at 100 µM but not at concentrations up to 50 µM. Error bars are ±SD (n = 3). b. Effect of Triton X-100 on the enhancement of PDK1 activity by RS2. The activity of PDK1 towards the peptide substrate T308tide was monitored by a radiometric assay as a function of RS2 concentration in the presence or absence of Triton X-100 detergent. Error bars are ±SD (n = 2). c. Effect of BSA on the enhancement of PDK1 activity by RS2. Same as B, except BSA was used in the place of Triton X-100.
Figure 3-S3. Density maps for PDK1-ligand complexes. Strong electron density was observed for the ligands (top panels $F_o$-$F_c$ simulated annealing omit maps) and for the residues lining the PIF pocket (bottom panels; $2F_o$-$F_c$ maps) in the a. PDK1:RS1, b. PDK1:RS2, and c. PDK1:PIFtide complexes. $F_o$-$F_c$ maps (green) were contoured to $3\sigma$ and $2F_o$-$F_c$ maps (blue) were contoured to $1.25\sigma$. PDK1 is colored cyan and the ligands are colored magenta.
Figure 3-S4. Mutation of Leu155 within the PIF pocket of PDK1 confers resistance to the diaryl sulfonamides.  

a. Dose-response curves for PIFtide and the RS compounds in a radioactive kinase activity assay that monitors the phosphorylation of T308tide peptide substrate by wild-type PDK1. Error bars are ±SD (n = 2).  

b. Same as a, except the mutant PDK1L155A is used.  

c. Same as a, except the mutant PDK1L155E is used.  

d. Effect of PIFtide and the RS compounds on the in vitro activation of S6K1 by PDK1L155A. Following activation of S6K1 by PDK1 for 30 min, the kinase activity of S6K1 was determined by a radioactive kinase assay using Crosstide substrate. The activity of S6K1 alone was used for normalization (dotted line). Error bars are ±SD (n=3).
Figure 3-S5. Structural comparison between the PDK1-PIFtide and AKT2-PIFtide complexes. 

**a.** Overlay of the PIFtide-binding poses of PDK1 (magenta) and AKT2 (grey, PDB: 1O6L). The root mean square deviation between these poses is 1.6 Å. 

**b.** Overlay of the PIF pockets of PDK1 (magenta) and AKT2 (grey). Residues that contact PIFtide in one or both structures are shown as sticks. Only 50% of these residues are identical between the two structures.
Figure 3-S6. RS1 selectively modulates the activity of PDK1 but not closely related kinases. The effect of 10 µM RS1 on the catalytic activity of 44 protein kinases was determined using the Invitrogen SelectScreen service. Kinases named in bold are the subject of our cell-based experiments. Error bars are ±SD (n = 2).
Figure 3-S7. The PIF-pocket ligand RS1 and the ATP-competitive inhibitor GSK combine additively to inhibit the activation of S6K1 *in vitro*. In this coupled assay, PDK1 first activates S6K1 for 15 min in the presence of drug or vehicle. Next the activity the S6K1 is measured by adding an S6K1-specific peptide substrate (Crosstide). The combined effects of a variety of doses of RS1 and GSK are displayed as a heat map above. The combination index (CI) for the two drugs at their respective IC50 values (CI<sub>50</sub>) is 0.93, reflecting that this combination is additive, as opposed to synergistic (CI<sub>50</sub> < 1) or antagonistic (CI<sub>50</sub> > 1).⁹
Figure 3-S8. Binding of ligands at the ATP-binding pocket and the PIF pocket is not cooperative. 

**a.** Titration of increasing concentrations of PDK1 against a fixed concentration of FP PIFtide probe. Addition of saturating nucleotide:Mg$^{2+}$ (0.5 mM) does not affect the binding affinity of the PIFtide. 

**b.** Titration of RS1 against a fixed concentration of PDK1 and FP PIFtide probe. Addition of saturating nucleotide:Mg$^{2+}$ does not significantly affect the apparent binding affinity of RS1.
Figure 3-S9. Original images of Western Blots. Phosphorylated AKT (T308) and S6 (S235-6) were detected using the Green (800) Li-COR secondary antibody, and α-tubulin was detected using the Red (700) Li-COR secondary antibody. The levels of the phospho-proteins and the loading control (α-tubulin) were measured simultaneously to reduce blot-to-blot variability.
Table 3-S1. Data collection and refinement statistics for complexes between PDK1 and diaryl sulfonamides

<table>
<thead>
<tr>
<th></th>
<th>PDK1+ATP+RS1</th>
<th>PDK1+ATP+RS2</th>
<th>PDK1+ATP+PIFtide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>C2</td>
<td>C2</td>
<td>C2</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>148.4, 44.2, 47.5</td>
<td>148.4, 44.2, 47.5</td>
<td>148.4, 44.2, 47.5</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90, 100.6, 90</td>
<td>90, 100.6, 90</td>
<td>90, 100.6, 90</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50-1.55 (1.61-1.55)</td>
<td>50-1.50 (1.55-1.50)</td>
<td>50-1.41 (1.46-1.41)</td>
</tr>
<tr>
<td>Rmerge</td>
<td>0.087 (0.918)</td>
<td>0.059 (0.556)</td>
<td>0.061 (0.832)</td>
</tr>
<tr>
<td>I / σ</td>
<td>14.7 (1.60)</td>
<td>18.5 (1.90)</td>
<td>18.9 (1.54)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.6 (97.1)</td>
<td>94.6 (65.8)</td>
<td>98.8 (91.5)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.4 (3.0)</td>
<td>3.5 (2.4)</td>
<td>3.5 (2.6)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>1.55 (1.61-1.55)</td>
<td>1.50 (1.55-1.50)</td>
<td>1.41 (1.46-1.41)</td>
</tr>
<tr>
<td>No. reflections</td>
<td>43970 (4160)</td>
<td>46184 (2900)</td>
<td>58386 (4501)</td>
</tr>
<tr>
<td>No. atoms</td>
<td>5170</td>
<td>5489</td>
<td>5414</td>
</tr>
<tr>
<td>Protein</td>
<td>4736</td>
<td>5013</td>
<td>5015</td>
</tr>
<tr>
<td>Ligand/ion</td>
<td>140</td>
<td>136</td>
<td>85</td>
</tr>
<tr>
<td>Water</td>
<td>294</td>
<td>340</td>
<td>314</td>
</tr>
<tr>
<td>Average B factors (Å²):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>20.0</td>
<td>20.2</td>
<td>20.9</td>
</tr>
<tr>
<td>Ligand/ion</td>
<td>35.1</td>
<td>34.1</td>
<td>29.4</td>
</tr>
<tr>
<td>Water</td>
<td>32.9</td>
<td>31.8</td>
<td>33.2</td>
</tr>
<tr>
<td>R.m.s. deviations:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.008</td>
<td>0.008</td>
<td>0.009</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.234</td>
<td>1.292</td>
<td>1.326</td>
</tr>
<tr>
<td>Ramachandran statistics (°) (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favored</td>
<td>97.9</td>
<td>97.7</td>
<td>98.4</td>
</tr>
<tr>
<td>Allowed</td>
<td>2.1</td>
<td>2.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Outliers</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*aValues in parentheses are for highest-resolution shell. bAs calculated by Molprobity.
Supplemental References


Chapter 4

Allosteric small-molecule modulators of the protein kinase PDK1 discovered by virtual screening.

T. Justin Rettenmaier*, Hao Fan*, Joel Karpiak, Allison K. Doak, Andrej Sali, Brian Shoichet, and James A. Wells
Abstract

Finding small molecules that target allosteric sites remains a grand challenge for drug discovery. In the protein kinase field, only a handful of highly selective allosteric inhibitors have been found. Thus, more general methods are needed to discover allosteric modulators for additional kinases. Here, we use virtual screening against an ensemble of both crystal structures and comparative models to identify ligands for an allosteric peptide-binding site on the protein kinase PDK1 (the PIF pocket). We optimized these ligands through an analog-by-catalog search that yielded compound 4, which binds to PDK1 with single-digit micromolar affinity. We confirmed the docking poses by determining a crystal structure of PDK1 in complex with 4. Because the PIF pocket appears to be a recurring structural feature of the kinase fold, known generally as the helix αC patch, our approach may enable the discovery of allosteric modulators for a number of additional kinases.
Introduction

Kinase inhibitors are essential research tools and valuable therapeutics. However, the majority of kinase inhibitors are not specific for the intended target, because they bind to the highly conserved ATP-binding pocket. The resulting off-target effects are often undesirable for a chemical probe and may cause side effects in patients. One approach to improve the specificity of kinase inhibitors has been to target allosteric sites distinct from the ATP-binding pocket, which are often less conserved among kinases. While this approach is still in its infancy, several striking successes have been reported and exquisitely selective allosteric inhibitors of the kinases AKT, MEK, and ABL are now in clinical trials for the treatment of advanced cancers.1 Despite these successes, developing allosteric inhibitors of protein kinases remains challenging, because the kinase of interest either has no known allosteric site or finding ligands for candidate sites is intractable with current technologies.

Here, we explored the druggability of an allosteric site called the helix αC patch. This site is functionally conserved across many evolutionarily distant protein kinases.2-4 The helix αC patch is a hydrophobic pocket formed by the αB/αC helices and the β4/β5 strands in the small N-terminal lobe of the kinase domain (Figure 4-1). The binding of effector proteins to the helix αC patch activates some kinases and inhibits others.2 We focused on the kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1) as a model system, because it is a paradigm for allosteric regulation by the helix αC patch. PDK1 is also an important anticancer target, because it phosphorylates and activates more than 20 kinases that regulate cell survival, proliferation, and metabolism, including isoforms of AKT, S6K, RSK, SGK, and PKC.5

PDK1 uses its helix αC patch, named the PDK1-interacting fragment (PIF) pocket, to recruit downstream kinases by engaging a hydrophobic peptide motif in their C-terminal tails.6 Because ATP-competitive inhibitors of PDK1 have so far been incapable of fully suppressing its activity7-9, small molecules targeting the PIF pocket have been pursued as a secondary strategy aiming to disrupt the recruitment of substrates to PDK1. A variety of computational and
experimental approaches have been used to target this challenging protein-peptide interface, including pharmacophore modeling\textsuperscript{10-12}, NMR-based fragment screening\textsuperscript{3, 13}, computational design\textsuperscript{14}, and competitive binding assays.\textsuperscript{4, 15} Here, we investigated the utility of virtual screening as a site-directed approach to discover new ligands for the PIF pocket of PDK1.

We performed virtual docking of 6300 compounds from the ZINC database against an ensemble of two crystal structures and four comparative models of PDK1. We prioritized compounds that scored well across at least four of the six target models. Using this consensus ranking approach, we identified two novel ligands, which were subsequently shown to bind to PDK1 with a $K_d$ of $\sim 40$ mM. Next, we docked commercially available analogs and discovered compound 4, which binds to PDK1 with an \textit{in vitro} potency that is comparable to known PIF pocket ligands ($K_d$ of 8 mM).\textsuperscript{4, 12} We solved a 1.4 Å crystal structure of PDK1 bound to 4, validating the predicted binding pose. In conclusion, we present new scaffolds for the development of allosteric PDK1 inhibitors and demonstrate the utility of virtual screening for targeting a challenging allosteric site that is present in a number of kinases.

Results

\textit{Generation of PDK1 comparative models and chemical library}

The virtual screening workflow is depicted in Figure 4-2. The conformation of the PIF pocket in PDK1 depends on the bound ligand. Specifically, the $\alpha$B and $\alpha$C helices were tightly packed against the kinase when PDK1 was bound to the allosteric activator \textit{PS48}, but the helices swung away from the kinase by up to 5 Å when PDK1 was bound to 1F8, a covalent PIF pocket ligand.\textsuperscript{3, 11} Moreover, Arg131 repositions to make optimal electrostatic contacts depending on which ligand is bound. Thus, we created a set of six structural models of the PIF pocket to recapitulate this repertoire of ligand-induced conformations. Model 1 (M1) is the crystal structure of PDK1 bound to \textit{PS48} (PDB 3HRF). M2 was constructed by grafting the $\alpha$B and $\alpha$C helices from the structure of PDK1 bound to 1F8 onto M1 (PDB 3ORX). We then relaxed the $\alpha$B and $\alpha$C
helices of M2 using MODELLER\textsuperscript{16} to compute M3, which is an intermediate between M1 and M2. Finally, we optimized the positioning of the Arg131 side chain using PLOP, thereby creating models M1R, M2R, and M3R (Figure 4-S1).\textsuperscript{17}

Next, we generated a chemical library for virtual screening. To avoid rediscovering known ligands, we assembled a list of 112 known PIF pocket ligands from the literature and our own work. We then used the DUD-E procedure to identify 6300 commercially available compounds that were topologically distinct from these known ligands but had similar chemical properties, such as molecular weight, calculated LogP, and net charge.\textsuperscript{18}

**Docking and experimental validation**

We performed a prospective virtual screen of the 6300 compounds against all 6 structural models using DOCK 3.6.\textsuperscript{19} To be considered a hit, we required that compounds rank in the top 500 in at least 4 of the 6 docking screens. We further triaged hits by imposing a geometry filter requiring that compounds must (1) make at least two hydrogen bonds to the polar subsite within the PIF pocket defined by residues Arg131, Lys76, or Thr148; (2) occupy the hydrophobic pocket delimited by Ile119 and Leu155; and (3) occupy the hydrophobic pocket with Phe157 at its base (Fig. 1B). The geometry filters were derived from crystal structures of PDK1 in complex with PIFtide or various small-molecule ligands. The polar subsite is a critical binding energy hotspot for a negatively charged carboxylate or aspartate. The hydrophobic pockets engage either two phenylalanines or two aryl substituents. Following visual inspection of the hit list, we selected three compounds for experimental testing (Table 4-1).

To determine whether the virtual screening hits bound to PDK1, we used a fluorescence polarization (FP) competitive binding assay that monitors the displacement of a fluorophore-labeled peptide from the PIF pocket of PDK1.\textsuperscript{4} Compounds 1 and 3 both bound to PDK1 with a $K_d$ of $\sim40$ mM, whereas the binding of 2 was negligible (Figure 4-3a). To confirm these findings with an orthogonal assay, we also determined the effect of 1 and 3 on the catalytic activity of
PDK1. Every non-covalent PIF pocket ligand reported to date stimulates the catalytic activity of PDK1 towards a short peptide substrate \textit{in vitro}. Accordingly, we found that compounds 1 and 3 maximally stimulated PDK1 activity by ~1.8-fold with an EC\textsubscript{50} of ~40 mM and ~50 mM, respectively (\textbf{Figure 4-3b}). Compounds 1 and 3 do not appear to exert their effects through an aggregation-based mechanism: Neither compound formed colloidal aggregates under our assay conditions, as monitored by dynamic light scattering, nor did either compound nonspecifically inhibit an unrelated enzyme – the protease cruzain (\textbf{Figure 4-S2}).\textsuperscript{20,21}

\textbf{Analog-by-Catalog}

To improve the potency of 1 and 3, we extracted 518 commercially available analogs from the ZINC database using its analog-by-catalog method with a permissive chemical similarity threshold of 70\% (Tanimoto coefficient, Tc).\textsuperscript{22} We docked these 518 analogs against the six structural models and found 15 analogs that scored as well as or better than the parent compounds 1 and 3 (\textbf{Table 4-S1}). We tested whether these analogs bound to the PIF pocket using the FP competitive binding assay described above. Of the 15 analogs tested, 8 bound to PDK1 with an affinity 2- to 4-fold worse than their parent compounds, 6 analogs showed very weak or negligible binding, and one (compound 4) bound to PDK1 with a \(K_d\) of 8 mM, corresponding to a 5-fold improvement over its parent compound 1 (\textbf{Table 4-2}). Compound 4 scored within the top 26 of 518 analogs across all six models and adopted a slightly different docking pose in each model (\textbf{Figure 4-S3}).

\textbf{Crystal Structure}

To assess the accuracy of the docking pose of compound 4, we determined a crystal structure of PDK1 bound to ATP and 4. To enable soaking of 4 into PDK1 crystals, we crystallized a double mutant of the kinase domain (Y288A, Q292G) that packs in an arrangement where the PIF pocket is accessible to the solvent.\textsuperscript{11} The best crystal diffracted to 1.4 Å resolution, and the
resulting electron density map showed strong peaks for both the aryl substituents and the carboxylic acid of 4 (Figure 4-4a). We did not observe clear electron density for the oxyethylsulfanyl linker, indicating it adopts multiple conformations. The only notable difference between the actual binding pose for 4 and the predicted docking pose is the conformation of the flexible linker between the aryl substituents (Figure 4-4b). The non-hydrogen atom RMSD of compound 4 between the predicted and experimental binding poses ranged from 0.92 to 1.56 Å following least-squares superposition of the six PDK1 models.

**Discussion**

Using structure-based virtual screening, we have identified novel ligands that bind to the PIF pocket, an allosteric peptide-docking site on the protein kinase PDK1. We improved upon the traditional single-structure virtual screening approach by docking against an ensemble of PDK1 structures and models. We hypothesized that ligands which dock well across multiple conformations of a binding site are more likely to be true binders. We also reasoned that docking against multiple conformations of the binding site would identify additional chemotypes compared to docking against a single structure. We were encouraged by previous ensemble docking studies, which have shown for a variety of protein targets that ensemble docking often increases the hit rate and chemical diversity compared to single target docking.\(^{23-25}\)

Here, our ensemble docking and consensus ranking approach showed three advantages over docking against a single crystal structure. First, we correctly identified the phosphonate 3 as a true binder despite its poor rank against the starting model M1 (2808/6300), because 3 scored well across the other 5 models. Moreover, a perturbed model prioritized 6 of the 9 true binders better than the crystal structures during the analog-by-catalog stage. Second, the success rate for identifying true binders exceeded 50% during both the initial screening (66%) and the analog-by-catalog steps (60%). Finally, using the consensus ranking approach to select hits greatly reduced the level of human intervention needed relative to our prior virtual screens.
against single targets. Therefore, docking against multiple models can lessen the need for an experienced scientist to scrutinize hundreds of docking poses, as is common practice in virtual screening.

We docked a library of only 6300 of the ~4 million commercially available compounds in the ZINC database. We selected this subset of compounds with the goal of retaining the chemical properties that are favorable for binding to the PIF pocket, the most notable being a net negative charge, while avoiding rediscovery of known chemotypes. This goal was accomplished by repurposing the DUD-E method, which was originally designed to identify property-matched “decoy” molecules as negative controls for docking. While the diaryl acid (1) and diaryl phosphonate (3) compounds can be broadly grouped into the same aromatic-charge-aromatic pharmacophore that describes nearly all reported PIF pocket ligands, both compounds represent novel scaffolds that are topologically dissimilar to all known ligands (maximum Tc of 0.25)\(^2\). Thus, they would not be discovered by searching for analogs of known ligands in the ZINC database using even a low 50% similarity cutoff. In essence, the DUD-E method for generating “negative control” molecules can surprisingly be repurposed for scaffold hopping when the protein target has a set of known ligands.

While 9 of the 15 compounds we selected during the analog-by-catalog step were true binders, only one analog (4) was significantly more potent than the parent compounds 1 and 3. This finding is consistent with common knowledge that computational docking is better at distinguishing true binders from non-binders than it is at predicting relative potencies. Thus, the success of an analog-by-catalog procedure is bounded by the number of analogs that can be purchased for testing and, indirectly, by the chemical diversity of the commercially available analogs. Nevertheless, we were able to identify analog 4, which was 5-fold more potent than its parent compound 1. The crystal structure of 4 bound to PDK1 revealed that the 2,6-dimethyl substituted phenyl group of 4 packs tightly into its hydrophobic subpocket, suggesting the 2,4-dimethyl substitution pattern of the parent compound 1 was sterically suboptimal. Additionally,
the poor electron density observed for the oxyethylsulfanyl linker between the phenyl and benzimidazole rings of 4 suggests this region is highly flexible. Therefore, in future studies the potency of 4 is likely to be further improved by rigidifying this linker with a carbocycle or other conformation-restricting moiety.

In conclusion, our findings demonstrate that virtual screening against the PIF pocket of PDK1 can identify novel allosteric ligands. The approach is also applicable to targeting the helix αC patch of other protein kinases, thus enabling the discovery of ligands for this broad class of protein–protein and protein–peptide interfaces.

Experimental Section

Generation of PDK1 structural models. Six structural models of human PDK1 were used in this study. The first model (M1) is the crystal structure of PDK1 bound to an allosteric activator (PDB 3HRF). Starting from the first structure, the PIF pocket allosteric site was adjusted in three steps to sample a variety of conformations. First, the positions of the αB and αC helices were extracted from another crystal structure with a disulfide-trapped fragment in the PIF pocket (PDB code 3ORX), resulting in the second structure (M2). Second, the αB and αC helices in M2 were refined using conjugate gradient (CG) minimization in MODELLER16, resulting in different positions of the αB helix and the loop that links the two helices (M3). Finally, the side chain of the Arg131 on the αC helix was optimized in all 3 models using the “sidechain prediction” protocol in PLOP, resulting in three more models (M1R, M2R, M3R).

Generation of virtual chemical library. With the goal of identifying novel ligands for the PIF pocket, a list of 112 known ligands was first compiled, consisting of 7 diaryl carboxylates and 105 diaryl sulfonamides. The docking library was then constructed from the ZINC database
using the DUD-E procedure\textsuperscript{18} to identify 6300 “decoy” compounds that had physicochemical properties similar to the known ligands but differed from them topologically.

**Virtual screening.** Virtual screening against the six PDK1 models was performed using a semi-automatic docking procedure. The receptor structure was prepared by removing all non-protein atoms from the crystal structures. Receptor-derived spheres were calculated using the program SPHGEN\textsuperscript{27} (part of the UCSF DOCK suite), while the ligand-derived spheres were generated from the positions of the heavy atoms of the crystallographic ligand in the 3HRF structure. In total, 45 matching spheres were used to orient ligands in the binding site. All docking calculations were performed with DOCK 3.6. The docking poses were scored using van der Waals, Poisson–Boltzmann electrostatic, and ligand-desolvation penalty terms.

**Chemical novelty evaluation and analog search.** For assessing chemical similarity between two compounds, we relied on the ECFP4 fingerprints\textsuperscript{26} to calculate Tanimoto coefficients (Tc) using the program Pipeline Pilot (Accelrys). A Tc value of less than 0.4 is commonly accepted as an indication of chemical dissimilarity between two compounds. Commercially available analogs of the initial docking hits were identified using the analog-by-catalog method of the ZINC database\textsuperscript{22} with a permissive chemical similarity level of 70%, as calculated by JChemBase (ChemAxon).

**Fluorescence polarization competitive binding assay.** Docking hits were experimentally tested for binding to PDK1 using a competitive binding assay that monitored the displacement of a fluorophore-labeled peptide from the PIF pocket.\textsuperscript{4} The dissociation constant ($K_d$) for ligands was calculated from their IC\textsubscript{50} values using an equation that accounts for ligand depletion.\textsuperscript{28}
**Cruzain assay.** Cruzain assays were performed in 100 mM sodium acetate, pH 5.5, containing 5 mM DTT. Triton X-100 was added to 0.01% in reaction mixtures as indicated. Drugs were incubated with 0.8 nM cruzain for 5 min until reactions were initiated by adding fluorogenic substrate Z-Phe-Arg-aminomethylcoumarin (Z-FR-AMC). The final reaction volume was 200 µL, containing 0.4 nM cruzain, 2.5 µM ZF-R-AMC, and 0.5% DMSO. Increase in fluorescence (excitation wavelength of 355 nm, emission wavelength of 460 nm) was recorded for 5 min in a microtiter plate spectrofluorimeter (Molecular Devices, FlexStation). Assays were performed in duplicate in 96-well plates; control samples contained DMSO only.

**Dynamic Light Scattering.** Concentrated DMSO stocks of drugs were diluted with assay buffer to a final DMSO concentration of 3.2%. Measurements were made using a DynaPro MS/X (Wyatt Technology) with a 55 mW laser at 826.6 nm, laser power of 100%, and detector angle of 90 degrees. Single-point measurements are reported.

**Protein kinase activity assay.** The effect of PIF pocket ligands on the catalytic activity of PDK1 towards a short peptide substrate (T308tide) was measured using a radioactivity-based kinase assay.³

**Crystallization and structure determination.** Crystals were obtained using a PDK1_{50-359} mutant (Y288G, Q292A) that disrupts a crystal contact that normally prevents ligands from binding to the PIF pocket.¹¹ Conditions for crystallization, compound soaking, harvesting, data collection, processing, structure building, and refinement were described previously.⁴ Diffraction data were collected at Advanced Light Source beamline 8.3.1. Final refinement statistics are summarized in Table 4-S2.
**Compound quality control.** Every purchased compound was analyzed by LCMS (Waters 2795 Analytical HPLC and ZQ MS). Every compound yielded a single peak by UV and evaporative light scattering (ELSD) and was within 0.1 Da of the expected mass. The structure of compound 4, 2-(2-((2-(2,6-dimethylphenoxy)ethyl)thio)-1H-benzo[d]imidazol-1-yl)acetic acid, was further confirmed by $^1$H-NMR (400 MHz, $d_6$-DMSO): $\delta$ 7.46-7.53 (m, 2H), 7.12-7.17 (m, 2H), 6.97 (d, $J$ = 7.6 Hz, 2H), 6.87 (dd, $J$ = 8.0, 6.8 Hz, 1H), 4.97 (s, 2H), 4.03 (t, $J$ = 6.4 Hz, 2H), 3.68 (t, $J$ = 6.0 Hz, 2H), 2.20 (s, 6H); LCMS ($m/z$): [M+H]$^+$ calcd., 357.12; found, 357.18.

**Acknowledgements**

We thank members of the Wells, Sali, and Shoichet labs for helpful suggestions and critical review of the manuscript. We also thank the staff at ALS Beamline 8.3.1. This work was supported by the NIH grants R01 CA136779 (J.A.W.) and U54 GM093342 (B.S., A.S., H.F.) and a NIH predoctoral fellowship (F31 CA180378) and Krevans fellowship (T.J.R.).
References


Figure 4-1. The relative position of the ATP-binding pocket and the helix αC patch on the protein kinase fold. a. PDK1 with a peptide bound to its helix αC patch, the PIF pocket b. Close-up view of the PIF pocket of PDK1. The hydrophobic sub-pockets targeted by docking are marked with two circles.
Figure 4-2. Virtual screening and experimental validation workflow.
Table 4-1. Top virtual screening hits across the six PIF pocket models.

<table>
<thead>
<tr>
<th>Compound structure&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Docking rank&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$K_d$&lt;sup&gt;c&lt;/sup&gt; µM (95% CI)</th>
<th>LE&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M1 M1R M2 M2R M3 M3R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6 39 29 66 1 2</td>
<td>39.1 (35.2-43.2)</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>109 189 31 297 14 76</td>
<td>&gt;200</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>2808 158 125 125 491 294</td>
<td>39.4 (36.8-42.2)</td>
<td>0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup> Charged states are depicted assuming a physiological pH of 7.4.

<sup>b</sup> Ranks reported do not consider the molecules discarded by the geometry filter.

<sup>c</sup> $K_d$ was calculated from the IC$_{50}$ in the FP assay using an equation that accounts for ligand depletion.<sup>28</sup>

<sup>d</sup> Ligand Efficiency (LE) is calculated as binding energy (DG, kcal/mol) per non-hydrogen atom.
Figure 4-3. Dose response curves for initial hits 1 and 3 and improved analog 4 in the a. binding and b. kinase activity assay. A peptide ligand was used as a control (PIFtide, residues 9-23).
Table 4-2. Structure-activity relationships for the PIF pocket ligand analogs.
Figure 4-4. The crystal structure of PDK1 bound to compound 4. a. Electron density is shown for the ligand (green, $F_o-F_c$ omit map, 3s) and for key interacting residues (blue, $2F_o-F_c$ map, 1.25s). b. Overlap of the crystallographic binding pose and the docking pose for compound 4, following least-squares superposition of the PDK1 atoms.
Figure 4-S1. Six PIF pocket models used for virtual screening.
Figure 4-S2. Experiments to rule out compound aggregation. Dynamic light scattering reveals no formation of colloidal particles by compounds 1 and 3 in the a. FP competitive binding assay buffer or b. kinase activity assay buffer. c. Cruzain enzyme activity assay demonstrates detergent-reversible inhibition by a known small-molecule aggregator, but no effect of compounds 1 and 3. Error bars are SD (n = 2).
Table 4-S1. Docking ranks and binding affinities for the 15 PIF pocket ligands.

<table>
<thead>
<tr>
<th>Compound structure</th>
<th>Docking rank</th>
<th>$K_d$ mM$^c$ (95% CI)</th>
<th>LE $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M1</td>
<td>M1R</td>
<td>M2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>45</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>389</td>
<td>238</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>90</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>433</td>
<td>188</td>
</tr>
<tr>
<td>9</td>
<td>60</td>
<td>375</td>
<td>95</td>
</tr>
<tr>
<td>Compound structure</td>
<td>Docking rank</td>
<td>$K_d$ mM$^c$ (95% CI)</td>
<td>LE $^d$</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------</td>
<td>------------------------</td>
<td>--------</td>
</tr>
<tr>
<td><img src="image1.png" alt="Diagram 10" /></td>
<td>M1 M1R M2 M2R M3 M3R</td>
<td>4 1 1 12 29 55</td>
<td>74 (62-87) 0.23</td>
</tr>
<tr>
<td><img src="image2.png" alt="Diagram 11" /></td>
<td>68 363 116 408 71 74</td>
<td>&gt;200</td>
<td>-</td>
</tr>
<tr>
<td><img src="image3.png" alt="Diagram 12" /></td>
<td>5 89 6 160 1 9</td>
<td>120 (100-140)</td>
<td>0.22</td>
</tr>
<tr>
<td><img src="image4.png" alt="Diagram 13" /></td>
<td>73 122 37 170 91 24</td>
<td>120 (110-130)</td>
<td>0.24</td>
</tr>
<tr>
<td><img src="image5.png" alt="Diagram 14" /></td>
<td>55 232 31 165 16 6</td>
<td>75 (66-85)</td>
<td>0.24</td>
</tr>
<tr>
<td><img src="image6.png" alt="Diagram 15" /></td>
<td>65 83 223 89 249 53</td>
<td>83 (76-90)</td>
<td>0.25</td>
</tr>
</tbody>
</table>
### Compound structure \(^a\)  Docking rank \(^b\)  \(K_d\) mM\(^c\) (95% CI)  LE \(^d\)

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M1R</th>
<th>M2</th>
<th>M2R</th>
<th>M3</th>
<th>M3R</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>63</td>
<td>410</td>
<td>470</td>
<td>2</td>
<td>405</td>
<td>115</td>
</tr>
<tr>
<td>17</td>
<td>271</td>
<td>306</td>
<td>407</td>
<td>7</td>
<td>466</td>
<td>59</td>
</tr>
<tr>
<td>18</td>
<td>412</td>
<td>221</td>
<td>468</td>
<td>164</td>
<td>455</td>
<td>447</td>
</tr>
</tbody>
</table>

\(^a\) Charged states are depicted assuming a physiological pH of 7.4.

\(^b\) Ranks reported do not consider the molecules discarded by the three geometry filters described in the text.

\(^c\) \(K_d\) was calculated from the IC\(_{50}\) in the FP assay using an equation that accounts for ligand depletion.\(^28\)

\(^d\) Ligand Efficiency (LE) is calculated as experimental binding energy (\(\Delta G\), kcal/mol) per non-hydrogen atom.
Figure 4-S3. The docking pose of compound 4 across the 6 PIF pocket models. The docking rank out of 518 analogs is shown at the bottom right of each panel.
Table 4-S2. Data collection and refinement statistics for the complex between PDK1 and compound 4

<table>
<thead>
<tr>
<th><strong>Data collection</strong></th>
<th>PDK1+ATP+RF4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PDB ID:</strong></td>
<td>4XX9</td>
</tr>
<tr>
<td><strong>Space group:</strong></td>
<td>C 1 2 1</td>
</tr>
<tr>
<td><strong>Resolution (Å):</strong></td>
<td>46.75-1.40 (1.45-1.40)</td>
</tr>
<tr>
<td><strong>Rmerge:</strong></td>
<td>0.065 (0.828)</td>
</tr>
<tr>
<td><strong>I/σ:</strong></td>
<td>18.9 (1.92)</td>
</tr>
<tr>
<td><strong>Completeness (%):</strong></td>
<td>99.7 (99.6)</td>
</tr>
<tr>
<td><strong>Redundancy:</strong></td>
<td>3.8 (3.6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Refinement</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resolution (Å):</strong></td>
<td>1.40 (1.45-1.40)</td>
</tr>
<tr>
<td><strong>No. reflections:</strong></td>
<td>59563 (5875)</td>
</tr>
<tr>
<td><strong>Rwork / Rfree:</strong></td>
<td>12.9 / 16.5</td>
</tr>
<tr>
<td><strong>No. atoms:</strong></td>
<td>5294</td>
</tr>
<tr>
<td><strong>Protein:</strong></td>
<td>4880</td>
</tr>
<tr>
<td><strong>Ligand/ion:</strong></td>
<td>138</td>
</tr>
<tr>
<td><strong>Water:</strong></td>
<td>276</td>
</tr>
<tr>
<td><strong>Average B factors (Å²):</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Protein:</strong></td>
<td>21.3</td>
</tr>
<tr>
<td><strong>Ligand/ion:</strong></td>
<td>30.6</td>
</tr>
<tr>
<td><strong>Water:</strong></td>
<td>33.6</td>
</tr>
<tr>
<td><strong>R.m.s. deviations:</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Bond lengths (Å):</strong></td>
<td>0.012</td>
</tr>
<tr>
<td><strong>Bond angles (°):</strong></td>
<td>1.468</td>
</tr>
<tr>
<td><strong>Ramachandran statistics (°):</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Favored</strong></td>
<td>98.3</td>
</tr>
<tr>
<td><strong>Allowed</strong></td>
<td>1.7</td>
</tr>
<tr>
<td><strong>Outliers</strong></td>
<td>0</td>
</tr>
</tbody>
</table>

*aValues in parentheses are for highest-resolution shell. †As calculated by Molprobity.*
Publishing Agreement
It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:
I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

_____________________________________   ______________________
Author Signature        Date

July 6, 2015

130