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

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Edited by Nikola P. Pavletich, Memorial Sloan Kettering Cancer Center, New York, NY, and approved November 17, 2014 (received for review August 9, 2014)

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 targeting a functionally conserved allosteric site on the protein kinase PDK1 called the PDK1-interacting fragment (PIF) peptide 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 (molecular weight ~300 Da) that compete with the peptide docking motif for binding to PDK1. We solved the first high-resolution structure of a peptide docking motif (PIF peptide) 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 PIFpeptide. 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 for exploring the helix Cα patches of other protein kinases as potential therapeutic targets even though they involve protein–protein interfaces.

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. Previous efforts to address these limitations have focused on targeting allosteric sites in kinases. Exquisitely selective allosteric inhibitors of the protein kinases AKT, MEK, and ABL are now in clinical trials for cancer, and various 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 Cα patch is an ancient allosteric site present on various serine/threonine and tyrosine kinases (2). The binding of effector proteins to the helix Cα patch activates some kinases and inhibits others. The helix Cα patch is seen 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 known role of PDK1 as a master regulator of these pro-survival and pro-growth 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 PIFpeptide, a synthetic peptide derived from the HM of the protein kinase PRK2, stimulates PDK1 activity toward a short peptide substrate (5) but disrupts recruitment and phosphorylation of the full-length substrates S6K and SGK (6). Small-molecule mimics of PIFpeptide have been discovered through pharmacophore modeling (7) and fragment-based approaches (8); and some optimized analogs have been characterized structurally (9–13); however, these compounds have limited membrane permeability, which diminishes their utility as chemical probes. Moreover, the lack of a structure of PIFpeptide bound to PDK1 has impeded the structure-based design of improved analogs that mimic the native allosteric interaction.

We have explored various site-directed methods for targeting the PIF pocket of PDK1. Previously, we used a technique known as disulfide trapping (or tethering) to identify small-molecule fragments (molecular weight <250 Da) that inhibit or activate PDK1 by covalently labeling a cysteine residue that was engineered into the PIF pocket (10). Here we sought to discover noncovalent small molecules that could be used as chemical probes of PIF pocket function in cells. We developed a PIFpeptide competitive binding assay to perform a site-directed screen of

**Significance**

Allosteric sites in protein kinases offer opportunities for developing more selective inhibitors, but these sites are challenging to target because they involve protein–protein interfaces. We designed a site-directed approach to screen for molecules that bind to an allosteric peptide docking site on the protein kinase PDK1. We discovered molecules that structurally mimic the natural peptide ligand and inhibit PDK1 in cells. We also found that combining allosteric and ATP-competitive inhibitors completely blocked the activation of oncogenic kinases downstream of PDK1. This approach could be adapted to target an analogous allosteric site found on many other kinases.

Author contributions: T.J.R., J.D.S., and J.A.W. designed research; T.J.R. and J.D.S. performed research; N.D.T., S.C.C., A.K.D., and M.R.A. contributed new reagents/analytic tools; T.J.R. analyzed data; and T.J.R. and J.A.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4RQK, 4RQV, and 4RRV).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1415365112/-/DCSupplemental.
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 (Fig. 1A). To achieve optimal sensitivity, FP competitive binding assays require a fluorescent probe that binds tightly to the target (KD < 100 nM) and yields a large change in polarization signal when bound (ΔmP > 100 mP) (14). To develop a suitable PIFtide probe for the FP assay, we synthesized a panel of fluorescent PIFtides of various lengths and amino acid content (full-length PIFtide: REPRILSEEEQEMFRDFDYIADW). 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 (KD = 40 nM; ΔmP = 130 mP; SI Appendix, Fig. S1). We used tetramethylrhodamine, a red-shifted fluorophore relative to fluorescein, to reduce the susceptibility of the assay to interference from autofluorescent compounds (15). The resulting competitive binding assay was suitably robust for a high-throughput screen (Z′ = 0.7).

The high-throughput screen workflow that we used is depicted in Fig. 1B and is discussed in detail in SI Appendix, Materials and Methods. In brief, 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 (1,460 hits; 0.9% hit rate). We filtered out autofluorescent artifacts by removing compounds that yielded total fluorescence intensities >150% of the untreated control. We tested the remaining 1,280 compounds using the FP assay in a 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 (7). 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 (Fig. 1C), which exhibited a KD value of 15 μM in the FP assay and an IC50 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 on 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 (Fig. 1C). RS1 and RS2 bound to PDK1 with a KD of 1.5 μM and 9 μM, respectively (Fig. 1D), and stimulated the catalytic activity of PDK1 toward a peptide substrate by twofold and sixfold, respectively (Fig. 1E). We further improved on the potency of RS-HTS by mimicking this peptide effector and providing insights into the structural 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 insights into 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.

Fig. 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 monitoring 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. After 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).
respectively (Fig. 1E). In comparison, δS-PIFtide bound with a $K_d$ of 2 μM and stimulated PDK1 activity by fourfold, whereas the diaryl dicarboxylate PS210 (13) bound with a $K_d$ of 3 μM and stimulated PDK1 activity by 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 <50 μM (SI Appendix, Fig. S2A), which is more than fivefold above their $K_d$ values. Moreover, the stimulation of PDK1 activity by RS2 was not diminished in the presence of Triton X-100 or BSA (SI Appendix, Fig. S2 B and C), additives 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.

Although RS1 and RS2 stimulated PDK1 activity toward a short peptide substrate, we expected to find that these PIF pocket ligands prevented PDK1 from phosphorylating most full-length protein substrates (e.g., S6K, SGK, and RSK), because the synthetic PIFtide bound to PKCδ has an affinity 100-fold less than that of full-length PIFtide (18). We expected PIFtide substrates to be efficiently phosphorylated in vitro (6) and in cells (18). 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 in vitro. Accordingly, we found that 20 μM δ8-PIFtide, RS1, and RS2 inhibited in the in vitro activation of S6K1 by PDK1 by 75%, 75%, and 60%, respectively (Fig. 1F).

**Structures of PDK1 Bound to Diaryl Sulfonamides.** To identify 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; Fig. 2A) or RS2 (1.5-Å resolution; Fig. 2B). Each structure showed unambiguous electron density for residues of varied length and obtained high-quality diffraction data (1.4-Å resolution; Fig. 2C). The RS compounds share a binding mode in which the aromatic substituents bind to two adjacent subsites in the PIF pocket. The sulfonamide group of both RS compounds interacts with Arg131 through a salt bridge, because 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 (SI Appendix, Fig. S4 A-C). The Leu155Ala mutation also conferred partial resistance to the RS compounds in the in vitro S6K1 activation assay (SI Appendix, Fig. S4D).

**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. To do so, 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 an unambiguous electron density for residues 13–21 of PIFtide (SI Appendix, Fig. S3 C) and demonstrates how PDK1 engages the core HM 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 ~90° 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 (Fig. 3A and B). Although the side chain of Asp16 could not be modeled, the Ca-Cβ bond vector for this residue appears to directly the side chain toward Arg131 on PDK1, suggestive of an electrostatic interaction. The conserved negatively charged residue of PIFtide (Asp18) interacts primarily with Gln150, not with Arg131 as was suggested previously (19). This charged binding mode is similar to that observed for the analogous Asp, phospho-Christian Teaching, and phospho-Thr in the HM of Akt (20), S6K1 (21), and PKCδ (22), respectively. Finally, Met13, Ile20, and Ala21 of PIFtide occupy small clefs at the periphery of the PIF pocket. A previously reported structure of an Akt chimera bound in cis to PIFtide (20) bears striking resemblance to the PDK1-PIFtide structure (PIFtide all-atom rmsd = 1.6 Å; SI Appendix, Fig. 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 is a proven reliable method for finding binding energy hot spots at protein–protein interfaces (23), and these hot spots often represent ideal small-molecule binding sites (24). We found that the strongly conserved residues at positions 14, 17, 18, and 19 of PIFtide all constituted binding energy hot spots (δδG of 1.5–2.5 kcal/mol), whereas non-conserved residues 10–13 and 15 contributed little to binding affinity (δδG of 0–0.75 kcal/mol) (Fig. 3C; each 1.4-kcal/mol increment in δδG represents a 10-fold loss in affinity). Although Asp16 is not strongly conserved among HMs, mutation to Ala significantly affected binding affinity (δδG of 1.25 kcal/mol), further supporting an electrostatic interaction with PDK1. These quantitative competitive binding data agree with previously reported qualitative immunoprecipitation binding data (25). In summary, the HM of PIFtide contains five amino acids (FxxFDYIA) that constitute binding energy hot spots (δδG >1.25 kcal/mol).

**Mimicry of PIFtide by the RS Compounds and PS210.** Comparing the binding modes of PIFtide and its small-molecule mimics revealed 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 (Fig. 4A) and the diaryl dicarboxylate PS210 (Fig. 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 Selectively to PDK1. 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 an HM in their respective PIF pockets. We also tested IGF1 receptor and mTOR, which directly impact signaling in cells. For these cell-based experiments, we used 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 (SI Appendix, Fig. S6). At 10 μM, RS1 did not inhibit 41 of the 44 kinases by >20%, suggesting 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) (26, 27) as a control for pathway modulation, and the diaryl dicarboxylate PS210 and its diester produg PS423 as a chemically distinct class of PIF pocket ligands (13).

We first tested whether RS1 would inhibit S6K1 activation in cells, as we found in vitro. We serum-starved HEK293 cells, treated them with increasing concentrations of RS1 or control compounds, and then stimulated them with IGF1 for 15 min before lysis. To observe the activation state of S6K1 in the cells, we monitored phosphorylation of its substrate ribosomal protein S6 by quantitative immunoblotting using infrared dyes. Treatment with increasing doses of RS1 led to a dose-dependent but incomplete blockade of S6 phosphorylation (Fig. 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 for efficient activation (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 had little effect on the phosphorylation of AKT (Fig. 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%, consistent with previous reports (27, 28). Control compounds RSi and PS210 did not affect AKT activation. In short, these findings indicate that 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 GSK in cells revealed that the activation of AKT by PDK1 is much less sensitive to inhibition by this ATP-competitive inhibitor compared with the activation of S6K, SGK, or RSK (27). The insensitivity of AKT activation is not explained by alternative pathways for AKT activation, because PDK1-deficient cells are incapable of activating AKT (18). Disrupting the capacity of AKT to bind to either PIP3 or the PIF pocket of PDK1 markedly sensitizes AKT phosphorylation to inhibition by GSK, suggesting that having multiple recruitment mechanisms contributes to insensitivity to GSK (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 them for 15 min with the growth factor IGF1 before lysis. We monitored...
prodrug of PS210. GSK, a selective ATP-competitive inhibitor of PDK1; PS210, a dicarboxylate PIF pocket ligand that does not enter cells; and PS423, a diester prodrug of phosphorylation was quantified from the infrared signal and normalized with α-tubulin signal. Error bars are = ±SD (n = 2). The following drugs were used: GSK, a selective ATP-competitive inhibitor of PDK1; PS210, a dicarboxylate PIF pocket ligand that does not enter cells; and PS423, a diester prodrug of S6 phosphorylation, reflecting the additivity of each compound’s effect (Fig. 6A and B) (27, 28). Cotreatment with RS1 and GSK resulted in enhanced inhibition of S6 phosphorylation, reflecting the additivity of each compound’s effect (Fig. 6A and B) (27, 28). However, even though RS1 did not block AKT activation by itself (Fig. 5B), it did enhance the ability of GSK to block AKT phosphorylation (Fig. 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 series of diaryl sulfonamide compounds that bind to the PIF pocket of PDK1 and disrupt its signaling in cells. Key to our success is 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 insights into 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 to find that both RS1 and PS210 bound to PDK1 with an affinity comparable to that of the 15-mer peptide Δ8-PIFtide (Fig. 1D). Alanine scanning mutagenesis revealed that five of the six residues within the HM of Δ8-PIFtide are critical for binding (Fig. 3C), yet RS1 and PS210 each lack a native electrostatic interaction (Asp18 and 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. The sulfonamides have a significant advantage over existing carboxylates, however, in that RS1 freely diffuses into cells, whereas PS210 does not. This disparity in cellular permeability may be attributable to the difference in pK₄ values between the carboxylic and N-arylsulfonamide moieties. At physiological pH, only a negligible fraction of carboxylate is protonated and thus cell-permeable (pK₄ ~3), whereas a significant portion of the sulfonamide is protonated (pK₄ ~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 (29). However, the purpose of an ester prodrug is to improve oral bioavailability of a drug by improving absorption in the gut (30). After its entrance into the circulation, PS423 would likely be rapidly hydrolyzed by esterases in the blood and liver (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.

Because PDK1 is an essential mediator of PI3K-AKT growth signaling, numerous PDK1 inhibitors have been developed as potential anticancer therapies (4). Two major findings diminished the enthusiasm for PDK1 as an oncology target, however. First, sustained knockdown of PDK1 levels by ~90% failed to prevent AKT activation or block tumor formation in PTEN-null mice (32). Second, GSK, the first potent and highly selective PDK1 inhibitor to be extensively characterized, did not significantly impact tumor growth in xenograft models (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 found that a small molecule that disrupts the recruitment of AKT by the PIF pocket of PDK1 induces sensitivity to an ATP-competitive inhibitor (Fig. 6B). The efficacy of this drug combination is not explained by R1 increasing the affinity of PDK1 for the active site inhibitor GSK, because binding at the ATP-binding pocket and the PIF pocket are not cooperative in vitro (SI Appendix, Fig. S8). Rather, the ability of the PIF-pocket ligand R1 to interfere with PDK1’s capacity to recruit substrates likely lowers the threshold of active site occupancy needed to effectively block PDK1 signaling with the ATP-competitive inhibitor GSK.

Taken together, these 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. Such a dual-targeting approach also may overcome the emergence of drug resistance, as has been demonstrated previously for targeting Ber-Abl in chronic myelogenous leukemia (33). Broadly, the site-directed approach described here could be adapted to target the helix cα 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. PDK1350, WT, Y228G Q292A, L155A, or L155E and 56K1241 T1412E were expressed in Sf9 insect cells using the Bac-to-Bac system. Proteins were purified by Ni-NTA affinity chromatography and size-exclusion chromatography. Details are provided in SI Appendix, Materials and Methods.

High-Throughput Screen. The overall screening strategy is depicted generally in Fig. 1B. Details are provided in SI Appendix, Materials and Methods.

**Chemical Synthesis.** Detailed methods for the synthesis and characterization of the R5 compounds, P5210, P5423, and the peptides are provided in SI Appendix, Note S1.

**Protein Kinase Activity Assays.** The catalytic activity of PDK1 toward the peptide substrate T308ptide (10) or the protein substrate S6K1 (6) were measured using radioactivity-based kinase assays as described previously.

**Crystallization, Data Collection, and Refinement of PDK1 Complexes.** Crystals were obtained using a PDK1350 mutant (Y228G Q292A) that disrupts a crystal contact that normally prevents ligands from binding to the PIF pocket (11). PDK1 was crystallized in complex with ATP, and crystals were soaked with ligand overnight before harvesting. Diffraction data were collected at Advanced Light Source beamline 8.3.1. More details on the crystallization conditions and structure solution are provided in SI Appendix, Materials and Methods. Final refinement statistics are summarized in SI Appendix, Table 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 before drug treatment for 1 h, stimulation with IGF1 for 15 min, and lysis. More details are provided in SI Appendix, Materials and Methods.

**Statistics.** All statistical analyses were performed using GraphPad Prism 6. All scatter plots bars and bar graphs depict mean values; all error bars are ± SD. Statistical significance was calculated using an unpaired two-sided t test assuming equal SD. The ic50 and ec50 values were calculated using a sigmoidal dose–response equation with variable slope. ic50 values from the FP competitive binding assay were converted to Kd values using an equation that corrects for ligand depletion (34).

**ACKNOWLEDGMENTS.** We thank members of the J.A.W. laboratory for helpful suggestions and critical review of the manuscript. We also thank the staff of Advanced Light Source beamline 8.3.1. This work was supported by National Institutes of Health Grant R01 CA136779-05 (to J.A.W.), a National Institutes of Health Predoctoral Fellowship F31 CA180378-01 (to T.J.R.), a Kevens 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.J.)].

**Conflict of Interest Statement.** This work was supported by a National Institutes of Health Predoctoral Fellowship F31 CA180378-01 (to T.J.R.). This work was supported by a National Institutes of Health Predoctoral Fellowship F31 CA180378-01 (to T.J.R.). This work was supported by a National Institutes of Health Predoctoral Fellowship F31 CA180378-01 (to T.J.R.). This work was supported by a National Institutes of Health Predoctoral Fellowship F31 CA180378-01 (to T.J.R.). This work was supported by a National Institutes of Health Predoctoral Fellowship F31 CA180378-01 (to T.J.R.).
Supplementary Information

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

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SI Materials and Methods:

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_{50-359} (wild-type, Y288G Q292A, L155A, or L155E) and S6K1_{24-421} T412E 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 (~2x10^6 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 (**Fig. 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 (1) 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(2). 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 (3). Structures were solved using molecular replacement with the structure of PDK1 bound to PS210 (PDB ID 4AW1) as a search model in Phaser (4). Iterative model building and refinement was performed with Coot (5) and PHENIX (6), respectively. Structure validation was performed using MOLProbity (7).

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; PKαβγ; PKCaβγδεθηιζ; 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 (8).

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 Na₃VO₄ (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.
Figure 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-CONH$_2$. 
**Figure 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 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 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 PDK1^{L155A} is used. (C) Same as A, except the mutant PDK1^{L155E} is used. (D) Effect of PIFtide and the RS compounds on the *in vitro* activation of S6K1 by PDK1^{L155A}. 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 S5. Structural comparison between the PDK1-PIFtide and AKT2-PIFtide (PDB: 1O6L) complexes. (A) Overlay of the PIFtide-binding poses of PDK1 (magenta) and AKT2 (grey). 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 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 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 of 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_{50}) is 0.93, reflecting that this combination is additive, as opposed to synergistic (CI_{50} < 1) or antagonistic (CI_{50} > 1) (9).
Figure 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 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 S1. Data collection and refinement statistics (molecular replacement)

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<td>148.4, 44.2, 47.5</td>
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<sup>a</sup>Values in parentheses are for highest-resolution shell. <sup>b</sup>As calculated by Molprobity.
SI Note S1:

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):

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

\[
\text{\text{H NMR (400 MHz, } d_6\text{-DMSO): } \delta 13.44 \text{ (broad s, 1H), 8.54 (s, 1H), 8.14 (d, } J=6.4 \text{ Hz, 1H), 8.10 (d, } J=6.4 \text{ Hz, 1H), 7.98 (d, } J=1.6 \text{ Hz, 1H), 7.53 (dt, } J=6.4, 0.8 \text{ Hz, 1H), 7.48 (td, } J=6.4, 0.8 \text{ Hz, 1H), 7.42 (dd, } J=7.2, 1.6 \text{ Hz, 1H), 7.27 (d, } J=6.4 \text{ Hz, 1H);} \]
\]
\[
\text{13C NMR (125 MHz, } d_6\text{-DMSO): } \delta 167.2, 140.0, 135.4, 134.8, 133.7, 133.6, 127.7, 127.4, 126.8, 125.6, 125.5, 123.4, 123.2, 122.5, 114.3; \]
\]
\[
\text{LCMS (m/z): [M+H]^+ calcd., 380.96; found, 381.0}
\]

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

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

\[
\text{\text{H NMR (400 MHz, } d_6\text{-DMSO): } \delta 8.05-8.0 \text{ (m, 4H), 7.49 (td, } J=7.2, 1.6 \text{ Hz, 2H), 7.44 (dd, } J=8.8, 2.4 \text{ Hz, 1H), 7.33 (d, } J=8.4 \text{ Hz, 1H);} \]
\]
\[
\text{13C NMR (125 MHz, } d_6\text{-DMSO): } \delta 168.0, 140.5, 137.4, 127.5, 127.4, 127.1, 125.7, 125.5, 123.0, 122.4, 114.7; \text{ LCMS (m/z): [M+H]^+ calcd., 380.96; found, 381.0}
\]

2-(3-oxo-1-phenyl-3-(4-(trifluoromethyl)phenyl)propyl)malonic acid (PS210):

\[
\text{2-(3-oxo-1-phenyl-3-(4-(trifluoromethyl)phenyl)propyl)malonic acid (PS210):}
\]

\[
\text{\text{H NMR (400 MHz, } d_6\text{-DMSO): } \delta 8.04 (d, } J=8.0 \text{ Hz, 2H), 7.86 (d, } J=7.6 \text{ Hz, 2H), 7.27 (d, } J=7.2 \text{ Hz, 2H), 7.20 (t, } J=7.6 \text{ Hz, 2H), 7.12 (t, } J=7.2 \text{ Hz, 1H), 3.87 (td, } J=10.8 \text{ Hz, 3.6 Hz, 1H), 3.74 (d, } J=10.8 \text{ Hz, 1H), 3.60 (dd, } J=17.2 \text{ Hz, 9.6 Hz, 1H), 3.42 (d, } J=17.2 \text{ Hz, 1H).} \]
\]
\[
\text{LCMS (m/z): [M+H]^+ calcd., 380.09; found, 380.1}
\]
bis(acetoxymethyl) 2-(3-oxo-1-phenyl-3-(4-(trifluoromethyl)phenyl)propyl)malonate (PS423):

\[
\begin{align*}
&\text{O} \quad \text{O} \\
&\text{O} \quad \text{O} \\
&\text{O} \quad \text{O} \\
&\text{F} \quad \text{F} \\
&\text{F} \quad \text{F}
\end{align*}
\]

$^1$H NMR (400 MHz, \textit{d}_6-DMSO): $\delta$ 8.03 (d, \textit{J} = 10.0 Hz, 2H), 7.88 (d, \textit{J} = 10.4 Hz, 2H), 7.28-7.34 (m, 2H), 7.23 (t, \textit{J} = 9.2 Hz, 2H), 7.16 (t, \textit{J} = 9.6 Hz, 1H), 5.72 (dd, \textit{J} = 14.4, 6.0 Hz, 2H), 5.45 (q, \textit{J} = 5.2 Hz, 2H), 4.19 (d, \textit{J} = 10.4 Hz, 1H), 3.94 (td, \textit{J} = 9.2, 4.0 Hz, 1H), 3.73 (dd, \textit{J} = 17.6, 9.6 Hz, 1H), 3.44 (dd, \textit{J} = 17.6, 4.0 Hz, 1H), 2.05 (s, 3H), 1.93 (s, 3H). LCMS (\textit{m/z}): [M+H]$^+$ calcd., 525.13; found, 525.1

References Cited: