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Design and synthesis of benzoazepin-2-one analogs as allosteric binders targeting the PIF pocket of PDK1

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ABSTRACT

A novel series of benzoazepin-2-ones were designed and synthesized targeting the PIF pocket of AGC protein kinases, among which a series of thioether-linked benzoazepin-2-ones were discovered to bind to the PIF pocket of 3-phosphoinositide-dependent kinase-1 (PDK1), and to displace the PIF peptide with an EC_{50} values in the lower micromolar range. The structure–activity relationships (SARs) of the linker region, tail region, and distal region were explored to further optimize these novel binders which target the PIF pocket of PDK1. When tested in an in vitro PDK1 enzymatic assay using a peptide substrate, the benzodiazepin-2-ones increased the activity of the enzyme in a concentration-dependent fashion, indicating these compounds act as PDK1 allosteric activators. These new compounds may be further developed as therapeutic agents for the treatment of diseases where the PDK1-mediated AGC protein kinases are dysregulated.

The activation of phosphatidylinositol 3-kinase (PI3K) by extracellular insulin and growth factors initiates intracellular protein phosphorylation cascades via the generation of the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3).[1] These signal events lead to the activation of a structurally related group of AGC (cAMP-dependent, cGMP-dependent, protein kinase C) family protein kinases, including protein kinase B (PKB)/Akt, p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK), serum- and glucocorticoid-inducible kinase (SGK), mitogen- and stress-activated protein kinase (MSK), isoforms of protein kinase C (PKC), protein kinase C-related kinase (PRK), and others. It is well established that AGC protein kinases regulate a diverse array of physiological processes relevant to cell metabolism, growth, proliferation, and survival[2] through distinct essential signaling pathways mediated by PI3K,[3–6] mitogen-activated protein (MAP) kinase[7,8] and Rho GTPase[9] and others. The deregulation of AGC family protein kinases has been found to be associated with human diseases such as cancer and diabetes.[10,11]

Despite the involvement in a variety of signaling pathways, the AGC protein kinases share two key regulatory features that are critical for their activation. First, their activation requires phosphorylation of a serine or threonine residue in the activation loop within the kinase domain, which results in a partial activation. For PKB/Akt,[12,13] S6K,[14,15] RSK,[16,17] SGK[5,6] and PRK,[9] the phosphorylation of their activation loops is catalyzed by 3-phosphoinositide-dependent kinase-1 (PDK1), an upstream master AGC kinase which phosphorylates and activates other members of the AGC kinase family. Second, most AGC family members also require the phosphorylation of a serine or threonine residue in a hydrophobic motif (HM) located in a conserved region at C-terminal of the kinase domains which results in the full activation.[12,13,18] In PKB/Akt, conventional PKCs, S6K and others, the HM is characterized by three aromatic amino acids surrounding the serine/threonine residue: Phe-X–X-Phe-Ser/Thr-Phe/Tyr; whereas in PRK and atypical PKCs, the same sequence is contained except that a negatively charged amino acid (aspartate or glutamate) mimics the phosphorylated serine/threonine.[19] With few exceptions, phosphorylation of both the activation loop and HM is required for the maximal activation of AGC protein kinases.

The mechanism by which HM phosphorylation triggers activation relies on the docking of the phosphorylated HM to a particular hydrophobic pocket in the N-terminal lobe of the kinase. Many

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AGC protein kinases contain this unique pocket which is formed by the αB and αC helixes and the adjacent β-sheet formed by β4 and β5. This HM binding pocket, referred to as the PIF (PDK1 Interacting Fragment) pocket, was initially determined to be the binding pocket by which PDK1 interacts with the phosphorylated HMs of downstream substrates, such as PRK2,[20] RSK2,[21] S6K, and SGK.[22,23] In addition, the binding of HMs can activate PDK1 and enhance phosphorylation of downstream kinases.[24] PDK1 harbors a PIF pocket similar to other AGC kinases, but lacks a complementary HM.[25] The intermolecular docking of a phosphorylated C-terminal hydrophobic motif (HM) of downstream kinases to the PIF pocket of PDK1 allows for the specific and selective control of AGC family protein kinases in response to extracellular events. Moreover, RSK, S6K, PKB/Akt, MSK and SGK also contain a HM/PIF pocket in the N-terminal lobe of the kinase, which are used to interact with their own C-terminal phosphorylated HMs.[26–29] The intramolecular docking of the phosphorylated HMs to the PIF pocket results in the ordering of the αB and αC helixes, which are disordered in the unphosphorylated and monophosphorylated forms of the kinase,[30,31] and subsequently induces synergistic stimulation of the kinase's catalytic activity. The HM phosphorylation site acts in concert with the activation loop phosphorylation site to stabilize the active conformation. Sequence conservation suggests that this mechanism is a key feature in the activation of more than 40 human AGC protein kinases.

It is possible that small molecules binding to the PIF pocket would regulate both PDK1 catalytic activity as well as interaction with substrates. Several small molecule allosteric activators of PDK1 have been reported recently from virtual screening and NMR-based fragment screening targeting the PIF pocket of PDK1 (Fig. 1).[31–34] These compounds are structurally similar and all contain a negatively charged carboxylate sandwiched by two aromatic ring hydrophobic moieties. The negatively charged carboxylate mimics the phosphate group of the phosphorylated HM and enables the interaction with phosphate-binding site adjacent to the PIF pocket; the two aromatic rings most likely mimic the two phenylalanine residues on the HM of the substrate kinases. Crystal structures have demonstrated that the identified small molecules could modulate the PDK1 activity through binding to the PIF pocket and prompting the conformational change both in the crystal and in solution. In an effort to identify selective small molecule modulators of PDK1, we performed virtual docking on the PIF pocket of PDK1 and a set of benzoazepin-2-one derivative scaffolds were designed de novo (Fig. 2). Molecular docking was carried out using GOLD [version 1.3 (Cambridge Crystallographic Data Center, Cambridge, U.K.)]. PDK1 crystal structure (PDB ID code 1H1W) was used as the protein template in docking. This molecular modeling study showed that these new scaffolds could bind to the PIF pocket mainly through hydrophobic interaction between the two aromatic groups and the PIF pocket and through the specific interaction of the tail substitute with the residues that form the phosphate-binding site which neighbors the PIF binding pocket. After the in silico evaluation of these scaffolds, benzoazepin-2-one, benzodiazepine-2-one, and pyrimidoazepin-2-one scaffolds were chosen as the starting points for further chemical elaboration. Based on their predicted binding modes (Fig. 2), SAR studies were conducted on the tail region, the linker region and the distal ring region.

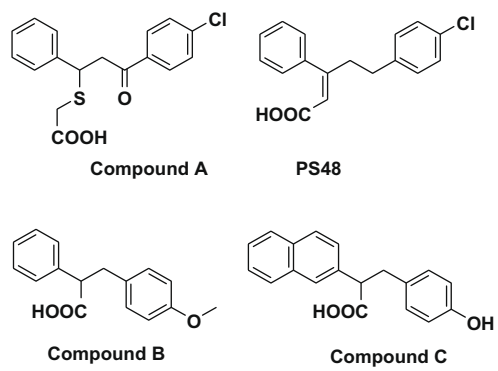


Figure 1. Small molecule allosteric activators of PDK1.

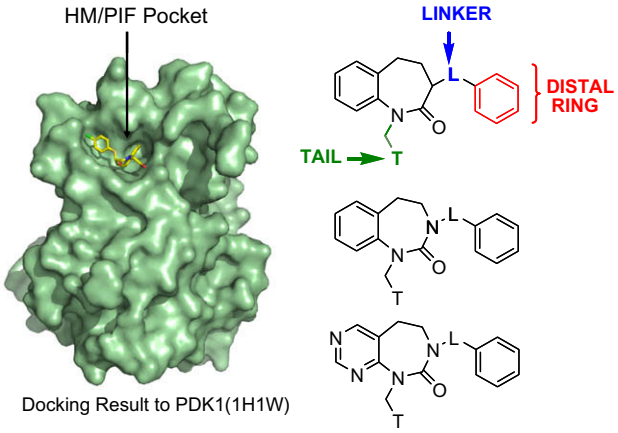


Figure 2. Predicted binding mode of benzo/pyrimido (di)azepine-2-one scaffolds (PDK1 coordinates from PDB ID code 1H1W).

The benzoazepin-2-one analogs were prepared by using commercially available 3-bromo-benzoazepin-2-one as the starting material (Fig. 3). Briefly, the treatment with sodium azide converted the bromide to azide. The reaction of the resulting azide and methyl bromoacetate afforded the N-alkylated intermediate. The reduction of the azide to amine followed by reductive amination of the amine intermediate with aldehydes yielded the amine-linked analogs. The corresponding amide- and sulfonamides-linked analogs were also synthesized by coupling with carboxyl acid and treatment with sulfonyl chlorides, respectively. Using a similar procedure, more linker modifications were explored; thus, 3-bromo benzoazepin-2-one was treated with phenol, thiophenol and carboxylic acid potassium salt, followed by N-alkylation, affording the corresponding ether-, thioether- and ester-linked analogs.

The synthesis of benzodiazepin-2-one analogs (Fig. 4) was straightforward. Starting from commercially available 2-(2-nitrophenyl) acetonitrile, the cyano group was reduced to amine, followed by the reductive amination with the corresponding aldehydes. Then the nitro group was reduced to amine. The ring-closure reaction was achieved by the treatment with triphosgene. Then N-alkylation afforded the corresponding benzodiazepine analogs.

The synthesis of the pyrimidoazepin-2-one scaffold began with treating 5-bromo-2,4-dichloropyrimidine with glycine methyl ester (Fig. 5). Stille coupling with allyltributylstannane, and oxidative cleavage of the terminal alkene by ozonolysis followed by in situ reductive amination afforded the key 4,5-di-substituted pyrimdine intermediate. This intermediate was then cyclised by the treatment with triphosgene and finally the amino groups were installed by the Pd-catalyzed amination to provide the corresponding pyrimidodiazepine-2-one analogs.

The ability (EC_{50}) of the de novo designed compounds to displace the PIFtide peptide[35] from the PIF pocket of PDK1 was then measured. Briefly, an HTRF assay measuring the binding of biotin-labeled PIFtide to the His-tagged PDK1 kinase domain protein was established using europim labeled anti His-tag antibody and allophycocyanin-streptavidin. Compound activity was measured

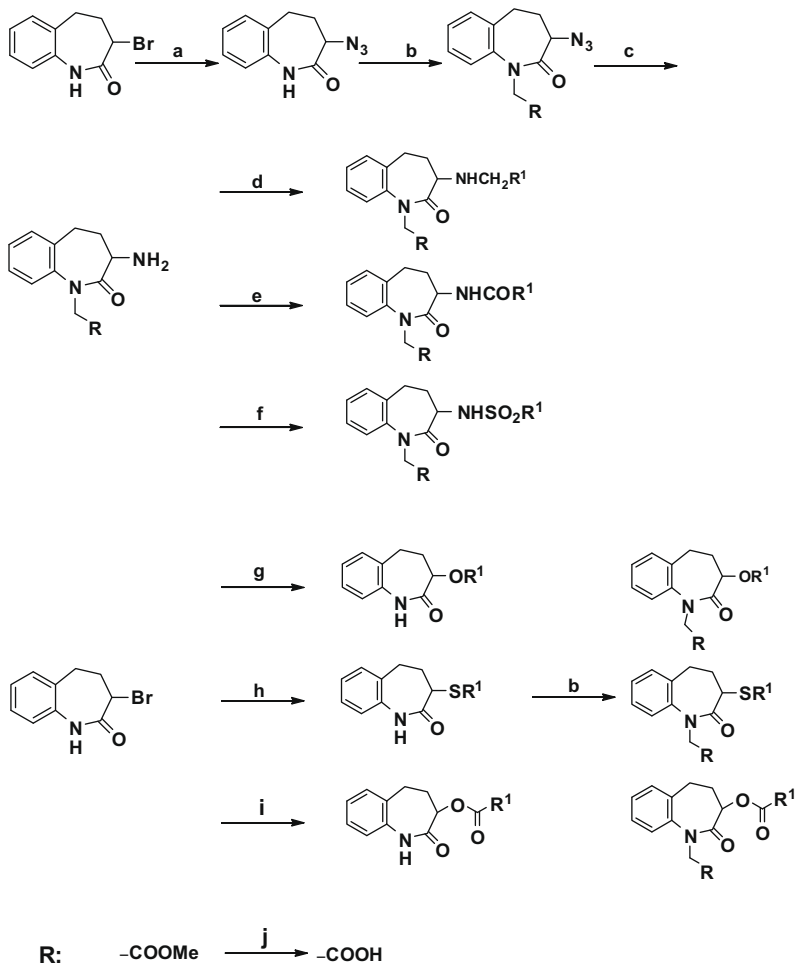


Figure 3. Synthesis of benzoazepin-2-one series: (a) NaN_3/DMSO, 80 °C; (b) methyl bromoacetate, $(Bu_4N)^+Br^-$, KOH/THF; (c) Pd/C, H_2/EtOH; (d) RCHO, $NaBH_4$/MeOH; (e) RCOOH, HATU, DIEA/DMF; (f) RSO_2Cl, DIEA/DCM; (g) ROH, NaH/dioxane; (h) RSH, NaH/dioxane; (i) RCOOK, 18-crown-6/MeCN; (j) LiOH/MeOH.

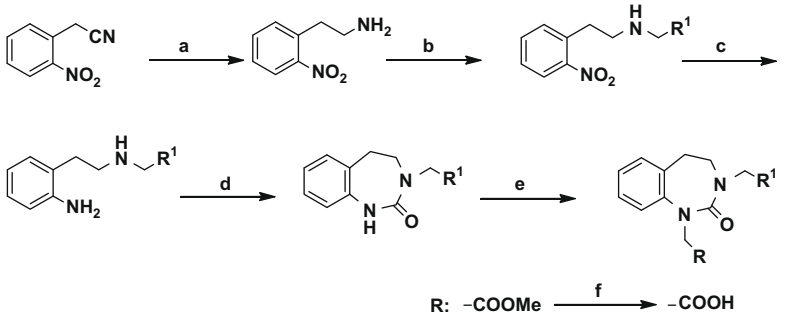


Figure 4. Synthesis of benzodiazepine-2-one series: (a) BH_3–THF (1.0 M); (b) RCHO, $NaBH_4$/MeOH; (c) Pd/C, H_2 /EtOH; (d) triphosgene/THF; (e) methyl bromoacetate, NaH/THF; (f) LiOH/MeOH.

in a competition assay and the compound concentration that resulted in 50% decrease of HTRF signal was designated as EC_{50}. Compound **A** (Fig. 1) was used as a positive control, as it is known to bind to the PIF pocket of PDK1 with an K_D value of 18 μM measured by isothermal titration calorimetry (ITC).[32] In our competition assay, the EC_{50} of this compound is 133 μM.

Based on binding assay results, it was found that the benzoazepin-2-one analog **2** bound to the PIF pocket of PDK1, whereas the benzodiazepine-2-one and pyrimidoazepin-2-one analogs showed weak or no detectable binding affinity (Table 1).

The terminal carboxylate (tail region) was required for the binding (Table 2). This observation could be explained by electrostatic interaction of the negative charge with Arg 131 of PDK1 (Fig. 6), consistent with the fact that the activation of PDK1 by the HMs of substrates is dependent on the phosphorylation of the HM or the acidic residue mimicking the phosphorylated site.[20,22]

The nature of the linker was also crucial for the binding. The thioether-linker showed the best binding affinity of all the linkers we explored though the amine linker also showed weak affinity (Table 2, **10** vs **2**). For the distal ring region (Table 3), a small

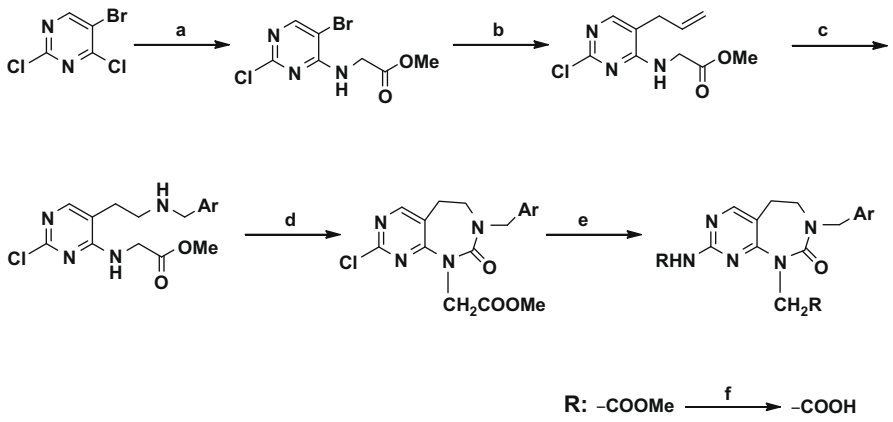
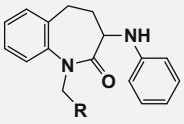
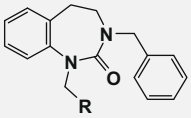
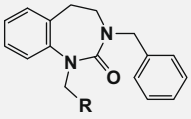


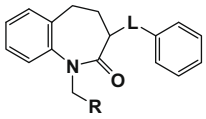
Figure 5. Synthesis of pyrimidodiazepine-2-one series: (a) $MeOOCCH_2NH_2$·HCl, DIEA/THF; (b) allyltributylstannane, $Pd(dba)_2$/tri-2-furylphosphine; (c) O_3, DCM then benzylamine, $NaBH_4$; (d) triphosgene, LHMDS/THF; (e) RNH_2, $Pd(OAc)_2$, Xantphos, dioxane, MW, 120 °C; (f) LiOH/MeOH.

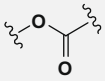
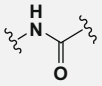
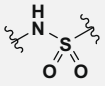
Table 1
Structures and binding affinities of compound **1–6**

Compd	Scaffolds	R	EC_{50}[a] (μM)
1		–COOMe	N/A
2		–COOH	900
3		–COOMe	N/A
4		–COOH	N/A
5		–COOMe	N/A
6		–COOH	N/A

[a] Values are means of three experiments, (N/A = not active).

Table 2
Structures and binding affinities of compound **1–2** and **7–16**



Compd	L	R	EC_{50}[a] (μM)
1		–COOMe	N/A
2		–COOH	900
7		–COOMe	N/A
8		–COOH	N/A
9		–COOMe	N/A
10		–COOH	101
11		–COOMe	N/A
12		–COOH	N/A
13		–COOMe	N/A
14		–COOH	N/A
15		–COOMe	N/A
16		–COOH	N/A

[a] Values are means of three experiments, (N/A = not active).

neutral substitution at the *para*-position was favorable. Thus, 2-(3-(4-chlorophenylthio)-2-oxo-2,3,4,5 tetrahydrobenzoazepin-1-yl) acetic acid (Table 3, **17**) was identified as the best binder with a EC_{50} value of 23 μM, showing a fivefold increase in binding affinity as compared to compound **A**, whose EC_{50} value was measured to be 133 μM in our assay as a reference.

To gain a functional insight into these PIF pocket binders, we tested compounds **17**, **18** and **21** in in vitro PDK1 enzymatic assay using a peptide substrate. As shown in Figure 7, these compounds increased the PDK1 enzymatic activity in a concentration-dependent fashion but to various degrees. At 25 μM, compounds **17** and **18** increased the PDK1 activity by 14-fold and 10-fold, respectively, whereas compound **21** showed less than twofold activation of the enzyme under the same conditions (Fig. 7). It does not appear there is a strong correlation between the fold increase of PDK1 activity and the EC_{50} value. Compound **18** binds to PIF pocket with the same affinity as compound **21**. Compound **18**, however, increased PDK1 activity more than fivefold higher than compound **21**. The 14-fold increase of PDK1 activity by compound **17** is the highest maximum activation reported by PDK1 PIF pocket activators. The highest concentration tested in the assay, 25 μM, was around the EC_{50} value of **17** binding to PDK1. We expect that **17** will increase PDK1 activity furthermore and the activation curve will reach a plateau at higher concentration. For comparison, the natural PDK1 activator, PIFtide, increases PDK1 activity up to about sevenfold.[32] Compound **A** was reported to increase PDK1 activity by approximately fourfold at high concentration (>200 μM).[31,32] These results demonstrate that small molecules binding to PIF pocket may induce slightly different conformational changes which lead to differential activation of PDK1 activity.

In summary, a series of novel benzoazepin-2-ones were designed to target the PIF pocket of PDK1 by de novo structure-based design. The SARs of the tail region, unlinker region, and distal phenyl ring region were explored and a sub-series of 2-thioether-benzoazepin-2-ones were identified to bind to the PIF pocket of PDK1. The designed compounds contain the common moieties identified in the known PIF pocket binders, that is, two aromatic rings and one carboxyl group. However, compared to known PIF pocket binders, the molecules presented here used a rigid cyclic benzoazepin-2-one scaffold to orient the aromatic rings and the carboxyl group that offers favorable binding entropy. This could explain the higher binding affinity of compounds **17** and **18** compared to compound **A**. In addition, the closely related molecules **18** and **21** that bind to PIF pocket with similar affinity caused different levels of activation; the further understanding of the molecular mechanism will require more structural studies including the co-crystallization of

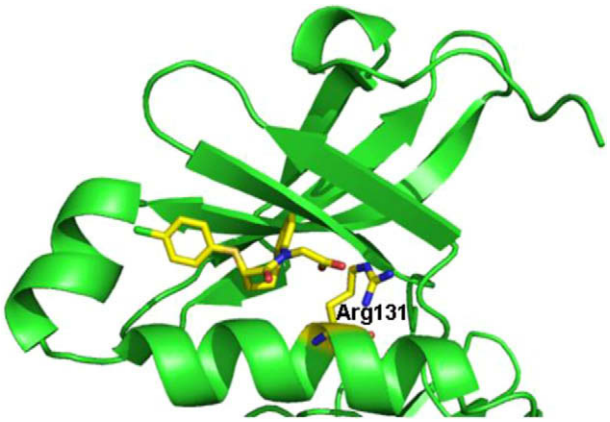


Figure 6. The proposed electrostatic interaction of the terminal carboxylate (negative charge) with Arg 131 (PDK1 coordinates from PDB accession code 1H1W).

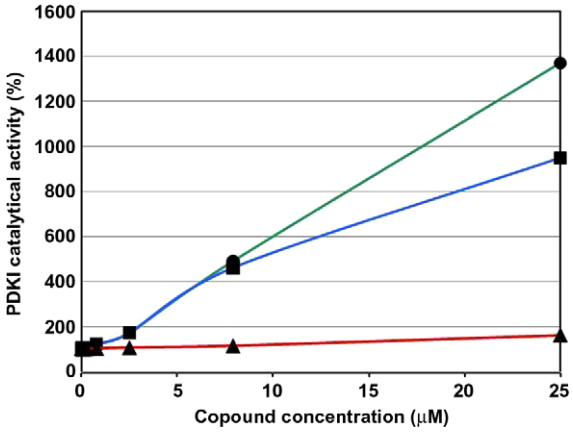
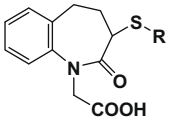
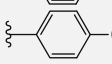
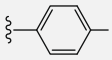
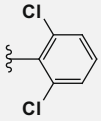


Figure 7. The PDK1 enzymatic assay. Compound **17** (circle), compound **18** (square) and compound **21** (triangle). The catalytic activity of PDK1 without compounds was set as 100%.

Table 3
Structures and binding affinities of compound **10** and **17–21**



Compd	R	EC_{50}[a] (μM)
10		101
17		23
18		53
19		187
20		75
21		45
22	Unlabeled PIFtide	10
A		133

[a] Values are means of three experiments.

these activators to PDK1. One of the designed compounds, compound **17**, showed comparable binding affinity to the unlabled PIFtide, a peptide derived from the natural PDK1 PIF pocket binding sequence.[24,33,35] The designed molecules activate the PDK1 activity in an in vitro enzymatic activity presumably by binding to the PIF pocket and allosterically stabilizing the active conformation. Compound **17** is the most potent activator of PDK1 reported so far in terms of the maximum fold increase of the PDK1 activity. A potent activator of PDK1 could be a valuable tool in studying the cellular signaling pathways involving PDK1. Activation of most downstream substrates of PDK1 such as S6K, RSK and SGK requires the binding of the substrates' phosphorylated HM motif to the PIF pocket of PDK1. The compounds identified here could potentially inhibit the activation of the downstream PDK1 substrates in the cell, and, therefore, they may have applications in inhibiting essential signaling pathways mediated by PI3K and provides a new strategy for drug discovery targeting this important pathway in cancer.

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References and notes

1. Cantley, L. C. *Science* **2002**, *296*, 1655.
2. Engelman, J. A.; Luo, J.; Cantley, L. C. *Nat. Rev. Genet.* **2006**, *7*, 606.
3. Ming, X. F.; Burgering, B. M.; Wennstrom, S.; Claesson-Welsh, L.; Heldin, C. H.; Bos, J. L.; Kozma, S. C.; Thomas, G. *Nature* **1994**, *371*, 426.
4. Franke, T. F.; Yang, S. I.; Chan, T. O.; Datta, K.; Kazlauskas, A.; Morrison, D. K.; Kaplan, D. R.; Tsichlis, P. N. *Cell* **1995**, *81*, 727.
5. Kobayashi, T.; Cohen, P. *Biochem. J.* **1999**, *339*, 319.
6. Park, J.; Leong, M. L.; Buse, P.; Maiyar, A. C.; Firestone, G. L.; Hemmings, B. A. *EMBO J.* **1999**, *18*, 3024.
7. Sturgill, T. W.; Ray, L. B.; Erikson, E.; Maller, J. L. *Nature* **1988**, *334*, 715.
8. Deak, M.; Clifton, A. D.; Lucocq, L. M.; Alessi, D. R. *EMBO J.* **1998**, *17*, 4426.
9. Flynn, P.; Mellor, H.; Casamassima, A.; Parker, P. J. *J. Biol. Chem.* **2000**, *275*, 11064.
10. Luo, J.; Manning, B. D.; Cantley, L. C. *Cancer Cell* **2003**, *4*, 257.
11. Taniguchi, C. M.; Emanuelli, B.; Kahn, C. R. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 85.
12. Alessi, D. R.; James, S. R.; Downes, C. P.; Holmes, A. B.; Gaffney, P. R.; Reese, C. B.; Cohen, P. *Curr. Biol.* **1997**, *7*, 261.
13. Stokoe, D.; Stephens, L. R.; Copeland, T.; Gaffney, P. R.; Reese, C. B.; Painter, G. F.; Holmes, A. B.; McCormick, F.; Hawkins, P. T. *Science* **1997**, *277*, 567.
14. Alessi, D. R.; Kozlowski, M. T.; Weng, Q. P.; Morrice, N.; Avruch, J. *Curr. Biol.* **1998**, *8*, 69.
15. Pullen, N.; Dennis, P. B.; Andjelkovic, M.; Dufner, A.; Kozma, S. C.; Hemmings, B. A.; Thomas, G. *Science* **1998**, *279*, 707.
16. Jensen, C. J.; Buch, M. B.; Krag, T. O.; Hemmings, B. A.; Gammeltoft, S.; Frodin, M. *J. Biol. Chem.* **1999**, *274*, 27168.
17. Richards, S. A.; Fu, J.; Romanelli, A.; Shimamura, A.; Blenis, J. *Curr. Biol.* **1999**, *9*, 810.
18. Testa, J. R.; Bellacosa, A. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 10983.
19. Parekh, D. B.; Ziegler, W.; Parker, P. J. *EMBO J.* **2000**, *19*, 496.
20. Balendran, A.; Biondi, R. M.; Cheung, P. C.; Casamayor, A.; Deak, M.; Alessi, D. R. *J. Biol. Chem.* **2000**, *275*, 20806.
21. Frodin, M.; Jensen, C. J.; Merienne, K.; Gammeltoft, S. *EMBO J.* **2000**, *19*, 2924.
22. Biondi, R. M.; Kieloch, A.; Currie, R. A.; Deak, M.; Alessi, D. R. *EMBO J.* **2001**, *20*, 4380.
23. Collins, B. J.; Deak, M.; Arthur, J. S.; Armit, L. J.; Alessi, D. R. *EMBO J.* **2003**, *22*, 4202.
24. Biondi, R. M.; Cheung, P. C.; Casamayor, A.; Deak, M.; Currie, R. A.; Alessi, D. R. *EMBO J.* **2000**, *19*, 979.
25. Balendran, A.; Casamayor, A.; Deak, M.; Paterson, A.; Gaffney, P.; Currie, R.; Downes, C. P.; Alessi, D. R. *Curr. Biol.* **1999**, *9*, 393.
26. Frodin, M.; Antal, T. L.; Dummler, B. A.; Jensen, C. J.; Deak, M.; Gammeltoft, S.; Biondi, R. M. *EMBO J.* **2002**, *21*, 5396.
27. Yang, J.; Cron, P.; Thompson, V.; Good, V. M.; Hess, D.; Hemmings, B. A.; Barford, D. *Mol. Cell* **2002**, *9*, 1227.
28. Huang, X.; Begley, M.; Morgenstern, K. A.; Gu, Y.; Rose, P.; Zhao, H.; Zhu, X. *Structure* **2003**, *11*, 21.
29. Smith, K. J.; Carter, P. S.; Bridges, A.; Horrocks, P.; Lewis, C.; Pettman, G.; Clarke, A.; Brown, M.; Hughes, J.; Wilkinson, M.; Bax, B.; Reith, A. *Structure* **2004**, *12*, 1067.

30. Yang, J.; Cron, P.; Good, V. M.; Thompson, V.; Hemmings, B. A.; Barford, D. *Nat. Struct. Biol.* **2002**, *9*, 940.
31. Hindie, V.; Stroba, A.; Zhang, H.; Lopez-Garcia, L. A.; Idrissova, L.; Zeuzem, S.; Hirschberg, D.; Schaeffer, F.; Jorgensen, T. J.; Engel, M.; Alzari, P. M.; Biondi, R. M. *Nat. Chem. Biol.* **2009**, *5*, 758.
32. Engel, M.; Hindie, V.; Lopez-Garcia, L. A.; Stroba, A.; Schaeffer, F.; Adrian, I.; Imig, J.; Idrissova, L.; Nastainczyk, W.; Zeuzem, S.; Alzari, P. M.; Hartmann, R. W.; Piiper, A.; Biondi, R. M. *EMBO J.* **2006**, *25*, 5469.
33. Stockman, B. J.; Kothe, M.; Kohls, D.; Weibley, L.; Connolly, B. J.; Sheils, A. L.; Cao, Q.; Cheng, A. C.; Yang, L.; Kamath, A. V.; Ding, Y. H.; Charlton, M. E. *Chem. Biol. Drug Des.* **2009**, *73*, 179.
34. Liu, Y. *Nature* **2009**, *461*, 484.
35. Labeled PIF tide: Biotin-KTFCGTPEYLAPEVRREPRILSEEEQEMFRDFDYIADWK-CONH2, was obtained from SynPep Corp. (Dublin, CA, USA). Unlabeled PIF peptide: QEMFRDFDYIADW, was synthesized by us and the purity verified by HPLC.