Discovery of PDK1 Kinase Inhibitors with a Novel Mechanism of Action by Ultrahigh Throughput Screening

Ekaterina V. Bobkova1‡, Michael J. Weber5, Zangwei Xu‡, Yan-Ling Zhang‡, Joon Jung‡, Peter Blume-Jensen‡, Alan Northrup1, Priya Kunapuli1, Jannik N. Andersen§, and Ilona Kariv‡

From the 1Merck Research Laboratories, Boston, Massachusetts 02115 and 5Merck Research Laboratories, North Wales, Pennsylvania 19454

The phosphoinositide 3-kinase/AKT signaling pathway plays a key role in cancer cell growth, survival, and angiogenesis. Phosphoinositide-dependent protein kinase-1 (PDK1) acts at a focal point in this pathway immediately downstream of phosphoinositide 3-kinase and PTEN, where it phosphorylates numerous AGC kinases. The PDK1 kinase domain has at least three ligand-binding sites: the ATP-binding pocket, the peptide substrate-binding site, and a groove in the N-terminal lobe that binds the C-terminal hydrophobic motif of its kinase substrates. Based on the unique PDK1 substrate recognition system, ultrahigh throughput TR-FRET and Alphascreen® screening assays were developed using a biotinylated version of the PDK1-tide1 substrate containing the activation loop of AKT fused to the peptide substrate-binding site, and a groove in the N-terminal lobe that binds the C-terminal hydrophobic motif of its kinase substrates. Using full-length substrate containing the activation loop of AKT fused to a pseudo-activated hydrophobic motif peptide. Using full-length substrate-containing the activation loop of AKT fused to a pseudo-activated hydrophobic motif peptide. Using full-length PDK1, $K_m$ values were determined as 5.6 μM for ATP and 40 nM for the fusion peptide, revealing 50-fold higher affinity compared with the classical AKT(Thr-308)-tide. Kinetic and biophysical studies confirmed the PDK1 catalytic mechanism as a rapid equilibrium random bireactant reaction. Following an ultrahigh throughput screen of a large library, 2,000 compounds were selected from the reconfirmed hits by computational analysis with a focus on novel scaffolds. ATP-competitive hits were deconvoluted by dose-response studies at 1× and 10× $K_m$ concentrations of ATP, and specificity of binding was assessed in thermal shift assay. Inhibition studies using fusion PDK1-tide1 substrate versus AKT(Thr-308)-tide and kinase selectivity profiling revealed a novel selective alkaloid scaffold that evidently binds to the PDK1-interacting fragment pocket. Molecular modeling suggests a structural paradigm for the design of inhibitory versus activating allosteric ligands of PDK1.

In cancer, oncogenic transformations are frequently associated with increased activity of protein-serine/threonine kinases, many of which are key signaling molecules in the phosphoinositide 3-kinase and mitogen-activated protein kinase (MAPK) pathways. Recent clinical studies have demonstrated that activating mutations in the MAPK pathway (i.e. K Ras and B Raf) confer resistance to anti-epidermal growth factor receptor antibody therapy (5, 6), suggesting that co-targeting of receptor tyrosine kinases and downstream serine/threonine kinases may be an attractive therapeutic strategy.

The serine/threonine protein kinases can be divided into two major families: the MAPK family, the members of which respond to the mitogen-activated G-protein RAS, and the extended AGC family of kinases (1). The latter comprises over 40 distinct human members including candidate oncology drug targets AKT, p70S6K, p90RSK, PRK3, protein kinase C, and PDK1. Many studies have focused on understanding the mechanism by which these AGC kinases are activated in cancer and following insulin and growth factor stimulation (7). It has been shown that the majority of AGC kinases are activated through the reversible phosphorylation at two highly conserved Ser/Thr residues (8). One is located in the kinase activation loop (also called the T-loop), whereas the other is located C-terminal to the catalytic domain, referred to as the hydrophobic motif (HM) or PDK1-interacting fragment (PIF) (9, 10). Phosphorylation of both residues is required for maximal activation. Specifically, PDK1 has been identified as the “master kinase” responsible for T-loop phosphorylation of AGC kinases and has thus attracted considerable interest as a candidate oncology drug target (7).

The PDK1 enzyme has evolved an intricate substrate recognition system that potentially can be harnessed as a target for small molecule ligands. Most prominently, in addition to the classical kinase active site, which binds ATP and the phosphoacceptor substrate residue, all AGC kinases contain a HM-binding pocket in the N-terminal lobe of the kinase domain, which specifically recognizes the phosphorylated HM (10, 11). In some AGC kinases, an acidic residue substitutes for the phospho-regulatory position of the HM, having the potential to mimic this activated conformational state (11, 12). Notably,

1 To whom correspondence should be addressed: 10901 N. Torrey Pines Rd., La Jolla, CA 92037. E-mail: ebobkova@burnham.org.

2 The abbreviations used are: MAPK, mitogen-activated protein kinase; PDK1, phosphoinositide-dependent protein kinase-1; uHTS, ultrahigh throughput screening; PIF, PDK1-interacting fragment; HM, hydrophobic motif; TR-FRET, time-resolved fluorescence resonance energy transfer; PH, pleckstrin homology; PRK, protein kinase C-related kinase; DMSO, dimethyl sulfoxide; DTT, dithiothreitol.
PDK1 is the only AGC kinase that lacks a HM in its C terminus. Therefore, the HM-binding pocket in the N-lobe of PDK1 is accessible to interaction with the phosphorylated HM of substrate kinases, and this intermolecular interaction promotes T-loop phosphorylation by PDK1 (13). Upon T-loop phosphorylation, the phosphorylated HM of the substrate kinase is released from the PIF pocket of PDK1, forming an intramolecular interaction with its own HM-binding pocket. This fully stabilizes the active enzyme conformation, resulting in phosphorylation of downstream effector molecules and downstream signal transduction (13).

The first evidence of this unique substrate recognition system was the discovery that PDK1 interacts with a C-terminal region of PRK2 in a yeast two-hybrid screen (14). The identified PIF contained a HM where the serine was replaced with a phosphoserine-mimicking aspartate (14). X-ray structural and mutagenesis studies have since shown that all PIF pocket residues critical for PDK1 substrate recognition, and a model “PIF-tide” has been generated based on the C-terminal HM of PRK2 (9). Conjugating the PIF-tide with the T-loop peptide from AKT(Thr-308)-tide resulted in a prevailing model PDK1 substrate, termed the “PDK1-tide,” that reportedly undergoes phosphorylation at a rate 100-fold greater than the AKT(Thr-308)-tide (9). More recently, small molecule PIF pocket binders were reported (15, 16), supporting the feasibility of developing allosteric modulators of PDK1 by interrogating this site.

To enable screening of vast ligand libraries and identify PDK1 inhibitors with novel mechanisms of action, including allosteric, we exploited the above substrate design paradigm and developed sensitive ultrahigh throughput enzymatic assays. Using a modified fusion peptide PDK1-tide1 that comprises a biotinylated signal enhancing sequence preceding the phosphorylation site fused to the PRK2 HM sequence, we screened a million chemical compound collection in the 1536-well format. In this paper, we describe the enzymatic properties of the full-length PDK1, screening assays, and uHTS campaign and report on the discovery of alkaloids as PIF pocket-specific ligands.

**EXPERIMENTAL PROCEDURES**

Reagents—Recombinant full-length human PDK1, expressed in Sf9 cells with a middle T tag at its N terminus (MEYMPME) and a molecular mass of 64 kDa, was from Kemp Biotechnologies (Frederick, MD). 5× kinase buffer and SYPRO® Orange protein stain (5000× solution in DMSO) were obtained from Invitrogen. AlphaScreen® general IgG (protein A) detection kit, white opaque 384-well Optiplates, and LANCE Eu-W1024-labeled anti-rabbit IgG antibody were purchased from PerkinElmer Life Sciences. Anti-phospho-AKT(Thr-308) monoclonal antibody was obtained from Cell Signaling Technologies (Danvers, MA). Streptavidin-linked DyLight fluorophore was purchased from Pierce. Streptavidin-APC was obtained from Prozyme (San Leandro, CA). 384-well black plates were from Greiner Bio-One GmbH (Frickenhausen, Germany). Flat bottom, nonbinding surface, nonsterile, white opaque 384-well Optiplates, and LANCE Eu-W1024-labeled anti-rabbit IgG secondary antibody, and 50 mM SA-DyLight. After 2 h of incubation at room temperature for an efficient antibody coupling, formation of the phospho-AKT product was detected on the Envision plate reader from PerkinElmer Life Sciences. A signal ratio at 615 nm/665 nm was used to calculate the percentage of inhibition, using no-ATP control as 100% inhibition.

Under the rapid equilibrium assumption, a random bisubstrate system is described as shown in Scheme 1. Thus, $K_{m}$ values can be determined from the slope replots derived from the double reciprocal plots by nonlinear regression using GraphPad Prism software (GraphPad Software, San Diego, CA) with the corresponding velocity equation (28).

$$
V_o = \frac{[A][B]}{\alpha K_A K_B} \\
V_{max} = \frac{1}{K_A + \frac{[B]}{K_B} + \frac{[A][B]}{\alpha K_A K_B}}
$$  

(Eq. 1)

**Development of the Fusion Peptide Substrate**—Several fusion peptide constructs were designed with a biotin group on either the N or C termini, using different linkers (see Table 1). Peptide synthesis and characterization was done at JPT Peptide Technologies GmbH (Berlin, Germany). Phosphorylation reactions were performed at 1 nM PDK1, 100 nM of the corresponding peptide, 5 μM ATP, 5 mM MgCl$_2$, and 2 mM DTT in 1× kinase assay buffer (10 mM HEPES, pH 7.5, 0.002% Brij-35, and 0.2 mM EGTA). To ensure linearity for approximation of the relative initial rates, kinetics were taken over the course of 180 min, and a TR-FRET signal reading at 30 min was selected for the comparative analysis of peptide substrate phosphorylation.

**Simultaneous Determination of $K_m$ Values for ATP and Peptide Substrates by Cross-titration**—Stock solutions of ATP and Pdk1-tide1, containing seven different concentrations of each substrate in 1× kinase buffer, were mixed at different ratios to generate 49 substrate mixtures with various concentrations of two substrates. The reactions were initiated by the addition of 15 μl of the enzyme mix, containing 0.5 nM of PDK1 enzyme in 1× kinase buffer, to the 15 μl of each substrate mix. The final substrate concentrations varied from 0.38 to 30 μM for ATP and from 10 to 650 nM for PDK1-tide1 peptide. To determine the initial reaction rates, kinetics were followed over the course of 60 min. At various incubation times, the reactions were stopped with the developing buffer, which contained 10 mM EDTA, 1 nM anti-phospho-AKT(Thr-308) primary monoclonal antibody, 5 nM LANCE Eu-W1024-labeled anti-rabbit IgG secondary antibody, and 50 nM SA-DyLight. After 2 h of incubation at room temperature for an efficient antibody coupling, formation of the phospho-AKT product was detected on the Envision plate reader from PerkinElmer Life Sciences. A signal ratio at 615 nm/665 nm was used to calculate the percentage of inhibition, using no-ATP control as 100% inhibition.
**Discovery of PDK1 Allosteric Ligands by uHTS**

5% (v/v) DMSO in a 40-µL reaction volume. The reaction was started with the addition of the ATP and stopped at 40 min by the addition of 20 µL of the developing buffer. TR-FRET was detected as described above, and the percentage of inhibition was calculated for each concentration. To determine the IC₅₀ values, dose-response curves were generated by nonlinear regression fit using a four-parameter binding model.

**1536-Well TR-FRET uHTS Assay**—For single-point screening in a 1536-well format, the final reaction conditions were 0.5 nM activated PDK1, 30 nM biotinylated fusion PDK1-tide1 peptide, 4 µM ATP, 5 mM MgCl₂, 100 mM NaCl, 2 mM DTT, 5% (v/v) DMSO, and 50 µM test compound in 1× kinase assay buffer with a total reaction volume of 4 µL. The first robotic dispense step was the addition of 1 µL of PDK1 enzyme solution in 1× kinase buffer containing 0.02% bovine serum albumin to a 1536-well, white, nontissue culture-treated polystyrene plate via a Kalypsys 8 tip bottle valve dispenser. Using the Kalypsys automated compound transfer pin tool, 50 nL of sample compound (suspended in 75% DMSO solution) and corresponding control compounds were added to the assay plate. After 10 min of preincubation with the compounds, the reactions were started by dispensing 1 µL of the substrate solution, containing ATP and PDK1-tide1 in 1× kinase buffer, with an additional Kalypsys 8 tip bottle valve dispenser. After 40 min of incubation at room temperature, the reactions were quenched by a 1-µL addition of EDTA (to a final assay concentration of 10 mM), followed by a 1-µL addition of detection antibodies (anti-phospho-AKT(Thr-308) primary monoclonal and LANCE Eu-W1024-labeled anti-rabbit IgG secondary antibody) to a final concentration of 3 nM each, along with 50 nM final concentration SA-DyLight via Kalypsys bottle valve dispensers. The plates were incubated for 1 h at room temperature to achieve optimal detection and then read on the Viewlux plate reader at excitation wavelength of 488 nm and an emission of 580 nm.

**Molecular Docking**—Docking of Alkaloid 1 ligand into the HM/PIF-binding pocket of PDK1 was performed using FRED, an exhaustive protein-ligand docking program from Openeye LLC. The binding site was defined by the key residues of Gln-150, Thr-148, and Ile-119 that outlined the HM/PIF-binding pocket in the high resolution x-ray structure of PDK1 (Protein Data Bank code 1UU3). FRED requires a precomputed data base of conformers; thus, OMEGA, a conformer generator from Openeye LLC, was used with default parameters to generate conformers. After exhaustive docking of conformers of the Alkaloid 1 by FRED, top scoring poses were filtered by visual inspection.

**RESULTS**

**Design and Evaluation of Biotinylated Fusion Peptide Constructs**—Several fusion peptides were synthesized and evaluated for their ability to serve as substrates for phosphorylation by PDK1 (Table 1). To develop proximity-based assays such as TR-FRET and AlphaScreen®, test peptides were biotinylated either at the N or C terminus. In contrast to the data generated using truncated PDK1 enzyme that lacks the C-terminal pleckstrin homology domain (ΔPH-PDK1) (9), we found that adding the PIF-tide to the enzymatic reaction does not stimulate but inhibits phosphorylation of both AKT-tide and PDK1-tide1 peptide substrates by a full-length form of PDK1 (Fig. 1). Because the fusion peptide PDK1-tide1, which contains a biotinylated enhancing linker sequence at its N terminus, showed a 4-fold higher signal than the AKT-tide, this construct was selected for further assay development.

**Development of High Throughput TR-FRET and AlphaScreen® Assays**—The determination of Kₘ values for ATP and fusion peptide substrates was performed in a 384-well format by using a simultaneous cross-titration of both substrates under non-saturating concentrations. The initial rates of kinase reaction were determined by kinetic measurements in the pres-
ence of various concentration combinations of both substrates. A $K_m$ value of $5.6 \pm 0.9 \mu M$ was obtained for ATP, and a $K_m$ value of $40 \pm 2 \text{ nM}$ was obtained for the fusion PDK1-tide1 peptide (Fig. 2).

To assure compatibility with the screening robotic protocols and to maximize the throughput, the incubation time of the kinase reaction in a 1536-well format was lengthened by lowering peptide substrate concentration to 30 nM, while keeping concentrations of other components identical to the 384-well plate assay. A robust signal generated with the PDK1-tide1 substrate afforded excellent 1536-well screening statistics with $Z'$ factor of $\sim 0.8$ (23).

When evaluated using reference kinase inhibitors (Fig. 3, compounds 1 and 2) (30–32), leads identified from the screen (compounds 3–5; structures not shown), and in-house tetracyclic (34) and tricyclic inhibitors (35), the sensitivity of the ultra-high throughput screening assay was comparable with the previously described 384-well AKT-tide based assay (33). To exclude false positive hits caused by the potential spectral artifacts, a secondary confirmatory assay in an AlphaScreen® format was developed for validation of the primary hits. Because AlphaScreen® is also a proximity assay, but with a chemiluminescent versus fluorescent type of detection, in a “red-shifted” wavelength range, the same biotinylated PDK1-tide1 substrate and detection antibodies can be used in this assay. The concentrations of the reaction components were maintained the same as in the TR-FRET assay to ensure comparable sensitivity toward the inhibitors.

**Development of the Thermal Shift Binding Assay**—To evaluate specificity of binding to the PDK1, we developed a follow-up thermal shift assay, based on monitoring the shift in the melting temperature ($T_m$) of the protein in the presence and absence of a bound ligand (17–19). The assay was validated with PDK1 ligands, including peptide substrates and reference kinase inhibitors, and with the newly developed tricyclic pyridone inhibitors of PDK1 (36) as an extended validation set (Fig. 4). The potency ranking obtained in the thermal shift assay for small molecule PDK1 inhibitors agreed well with the ranking in the PDK1 enzyme activity assay (Fig. 4, left panel). Based on the standard deviation of 0.4°C, a cut-off of 1.2°C (three times the S.D.) was chosen for the analysis of the thermal shift screen results.

Similarly, the relative affinity ranking of ligands, derived using a biophysical binding approach, agreed well with the enzymatic functional results (Fig. 4, right panel). A notable phenomenon was observed for the PIF-tide. While inhibiting phosphorylation of the AKT-tide substrate (Fig. 1), in the thermal shift assay it displayed a binding affinity similar to the PDK1-tide1. This suggests that most of the PDK1-tide1 binding energy is derived from the interaction with the PIF pocket. A rather moderate effect on the PDK1-tide1 phosphorylation can be explained by a suboptimally high (100 nM) concentration of the fusion substrate in the single dose experiment. When a dose-response inhibition study was performed at 30 nM of the fusion peptide (data not shown),
which is equivalent to \(1 \times K_m\), an inhibition of the PDK1-tide1 phosphorylation with the PIF peptide was observed with an \(IC_{50}\) value of 230 nM, thus confirming a competitive nature of the binding of these two peptides to the PIF pocket of PDK1.

**Ultrahigh Throughput Screening Strategy and Results**—Full-length PDK1 enzyme contains two domains: a C-terminal pleckstrin homology domain, which binds membrane-bound phospholipids, and an N-terminal kinase domain. The kinase domain comprises at least three ligand-binding sites in the vicinity of its catalytic site: the protein substrate-binding site, the ATP-binding site, and the PIF pocket. Several important considerations were taken into account when designing the uHTS strategy to identify structurally and mechanistically novel inhibitors of this kinase (Scheme 2). In principal, using a fusion peptide substrate would allow the discovery of inhibitors interfering with a number of these sites. Because unoptimized novel and allosteric inhibitors are likely to display low affinity, a high concentration of test compounds was used.

A corporate library of over a million compounds was screened in a 1536-well plate format at 40 \(\mu M\), resulting in 15,257 hits based on a cut-off of three times the S.D. (25% inhibition). Upon retesting in triplicate, 8,285 cherry-picks were reconfirmed in AlphaScreen\textsuperscript{®}, an alternative detection format, to filter out possible false positives caused by fluorescence artifacts. Of the resulting 7,013 reconfirmed hits, 2,051 compounds were excluded from further consideration because of the undesirable structural features and/or promiscuous inhibition of other HTS assays. Known PDK1 inhibitors belonging to the tetracyclic and tricyclic classes (33, 34, 36) were identified, thus validating the screening assays. To focus on the novel scaffolds, the next round of attrition included 720 hits that were structurally similar to known inhibitors of PDK1 (20). The structural clustering of the remaining hits generated 1,528 centroids with \(\sim 1,000\) singletons, resulting in a list of \(\sim 2,000\) structurally novel diverse hits selected for further follow-up.

To deconvolute ATP-competitive hits, a shift in an inhibitory potency was assessed by comparing \(IC_{50}\) values at 4 \(\mu M\) (1 \(\times \) \(K_m\)) versus 40 \(\mu M\) (10 \(\times \) \(K_m\)) concentrations of ATP. In parallel, all 2,000 compounds were also profiled in the thermal shift assay to prioritize for specific binders. Of 985 ATP-competitive hits identified, 460 compounds were confirmed as specific binders by thermal shift. 87 inhibitors, characterized as noncompetitive with ATP, had a thermal stabilization effect on the PDK1 protein. Among noncompetitive hits, two analogs with a micromolar level of inhibitory potency belonged to the alkaloid chemical series, with the tropine scaffold, as a common structural motif. When tested in the AKT-tide enzymatic assay, alkaloid analogs did not display a significant inhibitory activity, thus suggesting that binding of these compounds might occur exclusively at the PIF pocket as suggested by the molecular modeling (Fig. 5, A and B). Specific binding of alkaloid ligands to PDK1 was reconfirmed both by kinase inhibition in the PDK1-tide1 based assay and by the significant stabilization of the protein structure that resulted in a melting temperature shift comparable with the substrates.

To assess selectivity against other kinases, select hits were tested against a panel of 96 kinases at 5 \(\times \) \(IC_{50}\) test concentration. Although ATP-competitive hits displayed various degrees of nonselective behavior, an alkaloid series displayed a remarkable selectivity, with both analogs inhibiting less than 4% of 96 kinases when tested at 50 and 100 \(\mu M\), respectively (data not shown).

**DISCUSSION**

Since the commercial launch in 2001 of the first kinase drug Gleevec\textsuperscript{®} for the treatment of
chronic myelogenous leukemia, eight small molecule kinase inhibitors have received Food and Drug Administration approval, primarily for oncology applications. Given the central role of the phosphoinositide 3-kinase pathway in human diseases, including cancer and diabetes, compounds that modulate PDK1 activity may be useful as therapeutic agents. Indeed, the patent literature disclosed to date reflects a considerable pharmaceutical interest in targeting this enzyme (20). However, the overwhelming majority of the disclosed PDK1 inhibitors are relatively nonselective ATP analogs. Such kinase inhibitors, exemplified by UCN-01, target many other kinases with similar potency to PDK1 (21).

One approach that has been considered for the development of highly selective and potent kinase inhibitors is the rational design of bisubstrate analogs characterized by their ability to bind to two ligand-binding sites. Several examples of this approach applied to the insulin receptor tyrosine kinase and protein-tyrosine phosphatase 1B were previously described (25–27). Because PDK1 contains at least three ligand-binding pockets proximal to the kinase catalytic site, a bisubstrate ligand approach can in principal be exploited for this target if appropriate fragments could be identified. Therefore the primary goal of the HTS campaign was to develop a high throughput assay that would allow identification of both ATP-competitive inhibitors and PIF pocket binding allosteric ligands. This was achieved by developing a biotinylated peptide substrate (PDK1-tide1) that engages both the ATP- and PIF-binding pockets.

To ensure sensitivity of the ultrahigh throughput assay toward the inhibitors with different mechanisms of action, substrate levels have to be maintained at their respective $K_m$ values. Although a pseudo-first order approach is commonly used for evaluation of $K_m$ values, where the concentration of the substrate of interest is varied with the second fixed substrate present at the saturation level, the utility of this technique for kinase targets is limited by the practical issues of achievable peptide substrate levels both in the $K_m$ determination studies and during the screening campaign. In this work, we utilized a simultaneous cross-titration of both ATP and peptide substrates under nonsaturative conditions, as described for the bisubstrate enzyme systems in Ref. 28. The resulting high quality kinetic data allowed accurate $K_m$ value determination, thus ensuring sensitivity of the assay toward all types of inhibitors. Moreover, an important outcome of using this technique with the full-length enzyme was elucidation of the PDK1 catalytic mechanism as a rapid equilibrium random bireactant system, based both on the Lineweaver-Burk patterns and the thermal shift confirmation that all substrates are capable of binding the PDK1 enzyme independently of each other. This supports the previous detailed kinetic study for the truncated PDK1-ΔPH form of enzyme by Gao and Harris (11), where catalysis by PDK1 was consistent with either the rapid equilibrium random Bi-Bi system or a Theorell-Chance mechanism. At the same time, in contrast to the ΔPH-truncated PDK1 (11), we did not observe any negative binding cooperativity between ATP and PDK1-tide for the full-length enzyme, as judged by the reciprocal plots perfectly crossing at the horizontal axis.

Based on the direct $K_m$ comparison, the PDK1-tide1 peptide designed for the uHTS assay proved to be a 50-fold better substrate for the PDK1 kinase than the commonly used AKT-tide substrate. This is likely attributable to an enhanced binding caused by the presence of the PIF motif as evident from the thermal shift results. The much lower affinity of 70 μM for PDK1-tide previously reported using a manual radioactive approach (11) is likely due to utilization of the ΔPH truncated PDK1 enzyme. Although the full-length enzyme system used in our studies displayed more robust binding of the peptide substrates than in the studies with the truncated form of the enzyme (9, 11), our observation of a greater than 10-fold improvement in affinity to the PDK1-tide compared with the AKT-tide is consistent with previous reports (11).

To expand the mechanistic spectrum of the HTS hits, we used a full-length PDK1 enzyme to enable the potential identification of ligands that depend on the presence of an intact PH domain for binding, similar to the discovery of allosteric inhibitors of AKT (29). The importance of utilizing full-length enzyme when screening for allosteric modulators was further supported by our observation that the PIF-tide displays a modest inhibitory activity against the full-length enzyme, which is in contrast to observations using the ΔPH truncated kinase, where the PIF-tide is slightly stimulatory (9). In addition, a small molecule compound, reported in literature as an activator of the ΔPH truncated form of PDK1 with $AC_{50} = ~34 \mu M$ (15), was not effective at a concentration of up to 60 μM against the full-length kinase with either PDK1-tide or AKT-tide as substrates (data not shown), thus suggesting a likely difference in modulation of the PDK1 kinase activity when the PH domain is present.

Several independent studies demonstrated that the PIF pocket is amenable to small molecule binding (15, 16, 37, 38). Using a virtual screening approach of a ~60,000 compound chemical library, Engel et al. (15) identified several compounds that increased the activity of ΔPH-PDK1. Likewise, in a recent study, Stockman et al. (16) identified potential allosteric activators of the ΔPH-PDK1 enzyme using a NMR spectroscopic approach to screen a fragment library for compounds that bind to the PIF- and ATP-binding pockets. Both groups have used the truncated versions of PDK1 lacking a PH domain.

The uHTS study described here, utilizing a full-length PDK1 enzyme and a peptide substrate designed to mimic the impor-
tant physiological aspects of the PDK1 enzyme protein-substrate interaction, provided further validation for the PIF pocket as an amenable target for the small molecule modulators. Using the PDK1-tide1 substrate, we find that both the PIF-tide and the novel alkaloids have an inhibitory effect on the catalytic activity of the full-length kinase (Table 2). In contrast, despite the specific binding to PDK1 confirmed by the thermal shift, the alkaloids had no measurable effect on enzymatic activity when using the AKT-tide (IC\textsubscript{50} > 30 μM). Although the most likely reason is the relatively low affinity of these unoptimized scaffolds, one cannot exclude the possibility that these chemotypes do not modulate the PDK1 functional activity despite their specific binding. At the present time, because of the low affinity of the allosteric ligands, both those discovered in this work and those described in the literature (15, 16, 37, 38), any meaningful modulation studies in intact cells are deferred until more potent ligands are developed from these initial leads in the future.

To further evaluate the possible binding mode of Alkaloid 1, docking studies into the PIF pocket of PDK1 were performed using a high resolution PDK1 crystal structure previously reported in Ref. 35. The proposed mode of binding (Fig. 5, A and B) suggests that interaction between the alkaloids and PIF-binding pocket of PDK1 is mostly hydrophobic in nature. The benzyl moiety of Alkaloid 1 occupies a larger hydrophobic cavity outlined by Ile-118, Ile-119, Val-124, Val-127, and Leu-155, whereas the dimethylphenylamine moiety occupies an adjacent hydrophobic pocket outlined by Val-127 and Phe-157. Recently, a high resolution x-ray structure of PDK1 with a low molecular weight activator has been solved by Hindie et al. (37). The structure of PS48 bound to the PIF-binding pocket of PDK1 is mostly hydrophobic in nature. The benzyl moiety of Alkaloid 1 occupies a larger hydrophobic cavity outlined by Ile-118, Ile-119, Val-124, Val-127, and Leu-155, whereas the dimethylphenylamine moiety occupies an adjacent hydrophobic pocket outlined by Val-127 and Phe-157. Recently, a high resolution x-ray structure of PDK1 with a low molecular weight activator has been solved by Hindie et al. (37). The structure of PS48 bound to the PIF-binding pocket of PDK1 revealed that the phenyl ring of PS48 is interacting with a hydrophobic pocket comprising Ile-118, Ile-119, Val-124, and Val-127,
which is similar to interactions observed with the benzyl moiety of alkaloid compounds. Interestingly, the crystal structure revealed local conformational changes upon binding of PS48, which was different from the x-ray structure used in this study. Specifically, Phe-157, which is one of the residues outlining the second hydrophobic subpocket, rotated 90° such that the second phenyl ring of PS48 can be accommodated. Also, the side chain of Arg-131 shifted ~3.5 Å to accommodate a network of ionic and hydrogen bonds involving Thr-148, Arg-131, Lys-76, and Gln-150 with the carboxylate moiety of PS48. Hindie and co-workers (15, 37) highlighted the downstream effects these local conformational changes have on the overall allosterity of PDK1 structure and thus the activation mechanism seen with PS48 and similar analogs. The alkaloid compounds identified in this study do not contain carboxylate moiety; thus, it may be reasonable to hypothesize that the binding mode may differ, especially the region around Phe-157 and Arg-131, from that observed with PS48. The overlays between binding mode Alkaloid 1 and x-ray structure of PS48 bound to PDK1 are shown in Fig. 5C. Without the carboxylate group, which is critical for the binding and activation mechanism for PS48 and similar analogs (15, 37), alkaloid compounds may not induce similar local conformational changes and subsequently can elicit inhibition mechanism, as seen in this study. Interestingly, Engel et al. (15) examined the activity of PDK1 as well as a panel of AGC kinases containing a PIF-binding pocket with a close analog of PS48. It was shown that a close analog of PS48 can induce both activation and inhibition mechanisms depending on the subtle differences in the PIF-binding pockets of AGC kinases. This leads to the possibility that different mechanisms of activation (activation versus inhibition) of PDK1 can be achieved by different types of small molecules targeting the PIF pocket. Altogether, the molecular docking studies support our experimental observations that alkaloids bind to the allosteric PIF pocket as well as provide a structural basis for the inhibitory versus activation properties of this novel scaffold.

High selectivity of alkaloids demonstrated against an extensive panel of other kinases suggests that allosteric modulation might be a preferred approach to the development of more selective kinase inhibitors. Discovery of a novel alkaloid series of PIF pocket binders opens new avenues to the development of allosteric ligands for PDK1, which may lead to novel anti-cancer therapies as well as provide selective molecular tools for elucidation of the intricacies of the PDK1 pathway regulation in cells.

### TABLE 2
Properties of alkaloid analogs

| Compound | STRUCTURE | IC50, μM | ΔTm, °C |
|----------|-----------|----------|---------|
| Alkaloid 1 | ![Structure](image1.png) | 5.7 ± 0.4 | > 30 | 2.1 |
| Alkaloid 2 | ![Structure](image2.png) | 18.0 ± 1.3 | > 30 | 2.4 |

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