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An Allosteric Inhibitor Scaffold Targeting the PIF-Pocket of Atypical Protein Kinase C Isoforms

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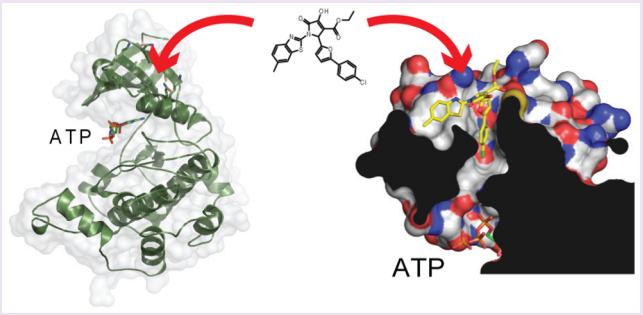
Supporting Information

ABSTRACT: There is a current and pressing need for improved cancer therapies. The use of small molecule kinase inhibitors and their application in combinatorial regimens represent an approach to personalized targeted cancer therapy. A number of AGC kinases, including atypical Protein Kinase C enzymes (PKCs), are validated drug targets for cancer treatment. Most drug development programs for protein kinases focus on the development of drugs that bind at the ATP-binding site. Alternatively, allosteric drugs have great potential for the development of future innovative drugs. However, the rational development of allosteric drugs poses important challenges because the compounds not only must bind to a given site but also must stabilize forms of the protein with a desired effect at a distant site. Here we describe the development of a new class of compounds targeting a regulatory site (PIF-pocket) present in the kinase domain and provide biochemical and crystallographic data showing that these compounds allosterically inhibit the activity of atypical PKCs. PS432, a representative compound, decreased the rate of proliferation of non-small cell lung cancer cells more potently than aurothiomalate, an atypical PKCι inhibitor currently under evaluation in clinical trials, and significantly reduced tumor growth without side effects in a mouse xenograft model. The druglike chemical class provides ample possibilities for the synthesis of derivative compounds, with the potential to allosterically modulate the activity of atypical PKCs and other kinases.



Despite remarkable advances in our understanding of the molecular mechanisms underlying cancer development and progression, a need for significantly improved therapies remains.[1,2] Protein kinases control and modulate a wide variety of biological processes, and deregulation of their activity has been implicated in many diseases, including cancer. Thus, during the past decade, numerous pharmaceutical programs focused their efforts on the development of small molecule kinase inhibitors.[3] The majority of the kinase inhibitors discovered to date target the ATP-binding site, which is structurally conserved across different families of protein kinases. As an alternative to the targeting of the ATP-binding site, selective modulation of protein kinase activity can be achieved by targeting the diverse allosteric sites that are involved in the regulation of protein kinases. The allosteric regulation of a given protein requires two distinct, distant sites, an orthosteric site and an allosteric site. Binding of ligands to the allosteric site induces a modification in the equilibrium of protein conformations, thereby affecting the functional orthosteric site and the cellular function of the target. Although there is great potential for drug discovery, challenges remain for the development of allosteric drugs against protein kinases and other drug targets.[4,5]

The group of AGC kinases, comprising more than 60 members in humans, is characterized by the presence of a hydrophobic pocket in the small lobe of the catalytic domain, termed the PIF-pocket, which is formed by helices αB and αC and strands β4 and β5, as first observed in the crystal structure of protein kinase A (PKA)[6] (SI Figure 1). In PKA, the PIF-pocket is constitutively occupied intramolecularly by the region C-terminal to the catalytic core comprising a truncated

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hydrophobic motif (HM). In other AGC kinases, the PIF-pocket plays a regulatory role; i.e., phosphorylation of the HM phosphorylation site of isoforms of PKB/Akt, S6K, RSKs, SGKs, PKCs, and MSKs triggers the intramolecular interaction of the HM with the PIF-pocket, supporting the stabilization of the active conformation of the catalytic domain.[7−11] In PDK1, the PIF-pocket serves a dual role, participating in a regulated docking interaction with the HM from substrates and, upon binding of the HM, participating in the allosteric activation of PDK1.[8,9,12−14] Besides phosphorylation, multiple other signaling inputs converge on the PIF-pocket to regulate the activity of AGC kinases. For example, the PH domain of PKB/Akt stabilizes a structure of the small lobe with a disrupted structure of the PIF-pocket.[15] The largest family within the AGC kinases in humans is the protein kinase C (PKC) family, members of which are classified into three structurally and functionally distinct subgroups: classical, novel, and atypical.[7,16,17] As members of the AGC kinase group, all PKCs have the HM, which occupies the PIF-pocket in the crystal structures of the active kinase domain of the PKC family.[18] In the case of atypical PKCs (aPKCs; PKCζ and PKCι), the residue that in related kinases is available for phosphorylation in the HM is substituted with Glu, a phosphomimetic amino acid. In addition, aPKCs possess a PB1 domain, which allows the interaction with other PB1 domain-containing proteins in heteromeric complexes.[16,17] Recent studies of atypical PKCζ and protein kinase C-related kinase 2 (PRK2) have also demonstrated that N-terminal domains can structurally affect the PIF-pocket and allosterically stabilize inactive conformations of the protein.[19,20] Furthermore, we also observed that the purified constructs of PKCζ, but not PKCι, formed large oligomeric structures in solution.[21] Additionally, we showed that helix αC from the PIF-pocket of PKCι is highly flexible in solution and is stabilized by the C1 domain that allosterically inhibits the activity of aPKCs.[21]

Interestingly, a number of low-molecular weight compounds that bind to the PIF-pocket were developed to allosterically stabilize the active conformation of the phosphoinositide-dependent protein kinase 1, PDK1,[13,14,22−29] thereby activating the kinase *in vitro*. Considerably less is known about the molecular mechanism of allosteric inhibition of AGC kinases by small compounds. A fragment compound covalently reacting with the Thr148Cys mutant of PDK1[7,27] provided evidence that the PIF-pocket could mediate partial allosteric inhibition of PDK1. Other allosteric inhibitors directed to PDK1[30] and PKCζ[31] have been described, although their binding to the PIF-pocket remains to be demonstrated. On the other hand, MK-2206 is a very selective allosteric inhibitor of Akt that binds to the PIF-pocket regulatory site in the inactive conformation, stabilizing a PH domain−catalytic domain intramolecular interaction.[32] We recently showed that a new compound, PS315, binds to the PIF-pocket in an overall active conformation and inhibits PKCζ by occupying a deep tunnel and subtly affecting the active site.[21] Recently, the "reverse" allosteric effect, initiated by compounds binding to the ATP-binding site and affecting the PIF-pocket regulatory site, has also been described in PDK1, adding examples to the bidirectional allosteric modulation of protein kinases with small compounds.[33]

Atypical PKCs are attractive targets for the treatment of cancer. Both isoforms have been found to be overexpressed in different tumor types and have been shown to play an important role in cancer development and progression.[34−38] PKCι is an oncogenic kinase contributing to the transformed phenotype of several types of cancer and is a validated target for lung and other cancers.[39,40] Inhibitors of aPKCs that interact with the ATP-binding site have been described previously.[36,41] In addition, the gold compounds aurothioglucose and aurothiomalate (ATM), which bind to the PB1 domain in aPKCs, were shown to have anticancer effects in *in vitro* and *in vivo* models of lung cancer.[42,43] These preclinical studies led to a phase I dose escalation clinical trial of ATM in patients with lung, ovarian, and pancreatic cancers. Phase II clinical trials are currently being conducted using another related gold compound, auranofin, for patients with chronic lymphocytic leukemia and in combination with sirolimus for stage IV non-small cell lung cancer patients (ref 44 and https://clinicaltrials.gov).

We here show the rational development of a series of druglike compounds that are allosteric inhibitors of aPKCs by targeting the PIF-pocket and occupying the deep tunnel. These molecules inhibit proliferation of cancer cells in culture and tumor growth in a lung cancer xenograft model. The novel compound class represented by PS432 shows synergy with a proteasome and PI3 kinase inhibitors, suggesting the potential future use of these allosteric compounds together with modern targeted drugs in personalized combination cancer treatments. In the meantime, different members of the scaffold presented can serve in the investigation of the role of the PIF-pocket in related protein kinases.

RESULTS AND DISCUSSION

Development of an Allosteric aPKC Inhibitor. We previously described the allosteric PDK1 activator PS114, as well as its variants that are allosteric inhibitors of PKCζ.[19] In an effort to improve the potency of allosteric inhibitors that target aPKCs, we rigidified the PS114 derivative PS155[23] by bridging positions 4 and 2 (Figure 1A). For the synthesis of the ethyl 4,5-dioxo-pyrrolidine-3-carboxylate derivatives PS309, PS312, PS212, and PS267, we used a three-component one-pot synthesis. These compounds were designed to possess a fixed angle between the benzoimidazole and benzothiazole rings, following the relative positions of the ring systems observed in crystal structures of allosteric activators and allosteric inhibitors bound to the PIF-pocket. Such a structure is generally not compatible with the flat structure required for binding to the ATP-binding site. PS212 and PS267 were of interest because they inhibited aPKCs in cell-free extracts (Table 1). In support of the specificity of the compound, we obtained the crystal structure of PS267 in complex with a PDK1−PKCι chimeric protein, which is based on PDK1 as a scaffold with eight mutations at the PIF-pocket to mimic the PIF-pocket of PKCι.[21] The crystal structure was determined to 1.41 Å resolution and refined to an R_{free} of 17.4% (Table S1). The structure of the complex showed that PS267 indeed binds to the PIF-pocket with the benzothiazole ring occupying a shallow subpocket and the thiophene ring sitting in a deep subpocket (Figure 1B,C). Only the ethyl ester moiety is unresolved, being solvent-exposed and apparently flexible. The mode of binding of PS267 to the PIF-pocket of the PDK1−PKCι chimera is similar to that of small compound activators of PDK1. Therefore, the mechanism by which PS267 allosterically inhibits aPKCs must involve an important effect on the molecular dynamics of the protein, stabilizing inactive conformations. At this point, the risk was that the further development of variants of PS267 would render activators

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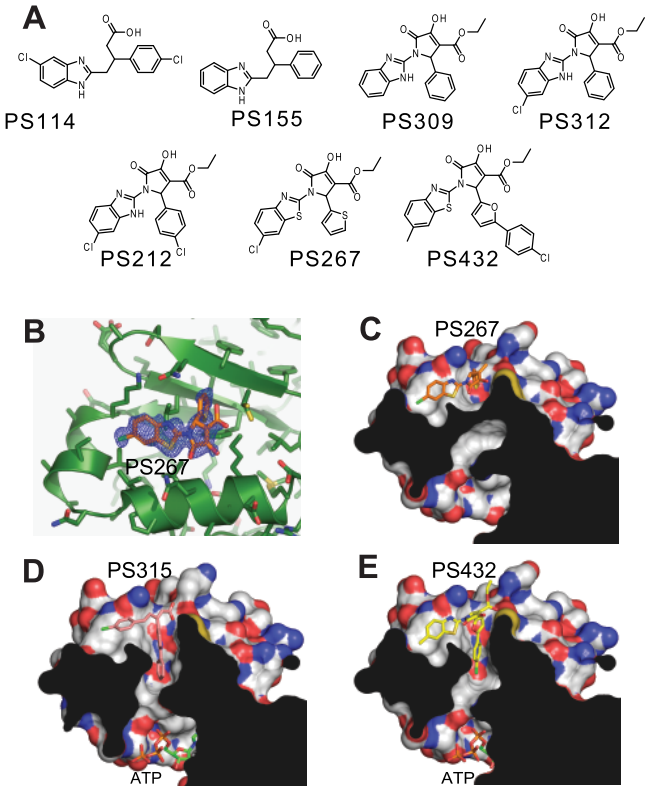

Figure 1. Chemical structures and modes of binding mode of PS compounds. (A) Chemical structures of PS compounds described in this study. (B) Crystal structure of PS267 bound to the PIF-pocket of the PDK1−PKC*ι* chimeric protein, comprising the PIF-pocket residues of PKC*ι* constructed on the scaffold of dmPDK1$_{50-359}$. (C) Cross section of the crystal structure depicted in panel B. (D) Cross section of the crystal structure of PS315 bound to the PIF-pocket of the PDK1−PKC*ι* chimeric protein showing the deep pocket. (E) Docked conformation of PS432 bound to the PIF-pocket of the PDK1−PKC*ι* chimera.

Table 1. Effects of PS Compounds Tested as Racemates on the *in Vitro* Activity of aPKCs

	IC$_{50}$ (*μ*M)	
	PKC*ι*	PKC*ζ*
PS114	ne[a]	ne[a]
PS155	ne[a]	ne[a]
PS309	ne[a]	ne[a]
PS312	ne[a]	44.1 ± 6.3
PS212	43.2 ± 0.2	20.5 ± 4.4
PS267	36.4 ± 0.8	17.8 ± 1.0
PS432	16.9 ± 0.3	18.5 ± 0.5

[a]No effect.

rather than inhibitors. On the other hand, PS267 did not make use of the deep tunnel that was occupied by PS315, a small molecule inhibitor of aPKCs described previously[21] (compare panels C and D of Figure 1). The mechanism of allosteric inhibition of aPKCs by PS315 suggests that other compounds occupying the deep tunnel would also be allosteric inhibitors. Therefore, to further develop PS267 derivative inhibitors, we decided to exploit the deep tunnel that had been uncovered in the crystal structure in complex with PS315. To fill the deep tunnel, we synthesized PS432, a derivative of PS267 with the tiophene ring substituted with a *p*-chlorophenylfuran moiety. Molecular docking studies of PS432 in the PIF-pocket of the PDK1−PKC*ι* chimera demonstrate a binding mode similar to that of PS267 but occupation of the deep tunnel as PS315 (Figure 1E).

Biochemical Characterization of PS432. PS432 inhibited PKC*ι* and PKC*ζ* with IC$_{50}$ values of 16.9 ± 0.3 and 18.5 ± 0.5 *μ*M, respectively (Figure 2A,B). PKC*ι* binds ROCKtide, an HM

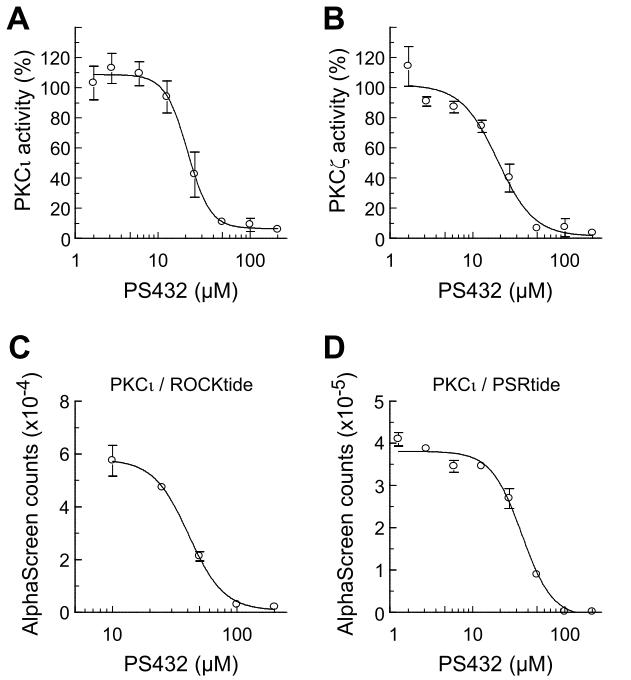

Figure 2. PS432 inhibits the kinase activity of atypical PKCs and displaces the hydrophobic motif and substrate peptide from their binding sites in the catalytic domain of PKC*ι*. (A) Effect of PS432 on the enzymatic activity of atypical PKC*ι*. An *in vitro* kinase assay was performed using the full-length recombinant PKC*ι* and MBP peptide as the substrate. (B) Same as panel A but using full-length recombinant PKC*ζ*. The results are presented as a percentage of the kinase activity relative to the vehicle control. The calculated IC$_{50}$ values are 16.9 ± 0.3 and 18.5 ± 0.5 *μ*M for PKC*ι* and PKC*ζ*, respectively. (C) Interaction displacement assay using Alpha-Screen technology. The interaction between PKC*ι* and ROCKtide (HM peptide), which binds to the PIF-pocket of PKC*ι*, was displaced in the presence of PS432 (IC$_{50}$ = 44 ± 3.8 *μ*M). (D) Alpha-Screen interaction assay showing the displacement of the interaction between PKC*ι* and PSRtide (pseudosubstrate peptide) that binds to the substrate-binding site of PKC*ι* by PS432 (IC$_{50}$ = 34.9 ± 3.1 *μ*M). Values represent the mean ± the standard deviation (SD) of three replicates, and the experiment was performed three times with similar results.

peptide that binds to the PIF-pocket, as well as PSRtide, a high-affinity pseudosubstrate peptide that binds at the peptide substrate-binding site.[21] PS432 was able to displace the binding of ROCKtide and PSRtide (Figure 2C,D). Together, these results indicate that PS432 binds to the PIF-pocket of PKC*ι*, displacing the HM peptide, and in addition promotes a conformational change that allosterically disturbs the high-affinity interaction of the pseudosubstrate with the peptide substrate-binding site.

Because PS432 binds to a site different from the active site of the kinase and in addition inhibits the intrinsic kinase activity of aPKCs, PS432 is an allosteric compound. Because PS432 is predicted to bind to the deep tunnel like PS315 does (Figure

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1E), we suggest that PS432 also disrupts the salt bridge between Lys111 and Glu130, disturbing the position of the phosphates of ATP and allosterically affecting the catalytic mechanism of the kinase.

PS432 was also evaluated against other PKC isoforms as well as representative members of the AGC group of protein kinases. At the concentration tested, PS432 did not inhibit the activity of PKCα, PKCβ, PKCδ, or PKCθ (Table S2) or the activity of related kinases such as PDK1, PKB/Akt, RSK1, MSK1, and Aurora A (Table S3). The results of these assays also demonstrated the activity of PS432 against two other kinases (SGK and S6K) in addition to aPKCs. However, the activity of these two potential off-target kinases was not affected in a cellular context, as we did not observe any changes in the phosphorylation state of their substrates in various cancer cell lines treated with 25 μM PS432 over a 24 h time period (SI Figure 2). The mutant forms of S6K (S6K-T2-[412E]) and SGK (SGK-[422D]) that were inhibited *in vitro* by PS432 have Glu and Asp, respectively, instead of the HM phosphate present in the native active kinases. It is possible that Asp and Glu do not fully mimic the effects of the phosphorylated HM on their corresponding PIF-pockets and phosphate-binding sites. In such a scenario, we suggest that the *in vitro* inhibition of the mutant kinases could be due to the increased dynamics of helices αB and αC facilitating the displacement of the mutant HM from the PIF-pocket and the subsequent PS432 binding and inhibition. At any rate, the finding that these two mutant kinases can also be inhibited by PS432 *in vitro* supports the notion that derivatives of PS432 may be developed to become specific inhibitors of other kinases. In *Saccharomyces cerevisiae* and related yeasts, the Pkh-Pkc axis (PDK1-PKC) is upstream of the Bck1 (MEKK) signaling, controlling the cell wall integrity (CWI) pathway.[45] PS432 was toxic to the control yeast cells, but the toxicity was reduced when a constitutively active Bck1 kinase was overexpressed (SI Figure 3), indicating that the effect of PS432 was not due to a general toxic effect.

Activity of PS432 in Cancer Cells. PS432 inhibited the anchorage-dependent proliferation of lung cancer cell lines A549 (IC_{50} = 14.8 ± 4.2 μM) and A427 (IC_{50} = 10.4 ± 0.3 μM) (Figure 3A) as well as androgen-independent prostate cancer cell line DU145 (IC_{50} = 20.8 ± 9.0 μM) (SI Figure 4A). Interestingly, growth of normal prostate epithelial cells (PNT1A) was not affected under conditions that reduced the rates of A549 and DU145 cancer cell proliferation by more than 80 and 60%, respectively (SI Figure 4B). These findings indicated that PS432 has potential as a chemotherapeutic agent with low toxicity. We further investigated the effect of PS432 on anchorage-independent cell growth, a phenotypic hallmark of neoplastic transformation, using a one-week 96-well soft agar colony formation assay.[46] PS432 inhibited the proliferation of A549, A427, and DU145 cells in semisolid agar medium with IC_{50} values of 30.7 ± 0.6, 12.4 ± 0.6, and 19.8 ± 1.9 μM, respectively (Figure 3B and SI Figure 4C). Under these assay conditions, PS432 reduced the rate of growth of lung cancer cell lines in soft agar more potently than ATM did (Figure 3B), a PKCι inhibitor currently being evaluated in clinical trials. The weaker effect of ATM in cells is not unexpected because ATM blocks the PB1 domain but does not inhibit the activity of the enzyme. Therefore, it is possible that ATM only blocks the phosphorylation of a subset of substrates of aPKCs.

It has been described that PKCι regulates the expression of RCF4, ELF3, PLS1, and COPB2 genes in lung cancer cell lines, and knockdown of PKCι leads to inhibition of their

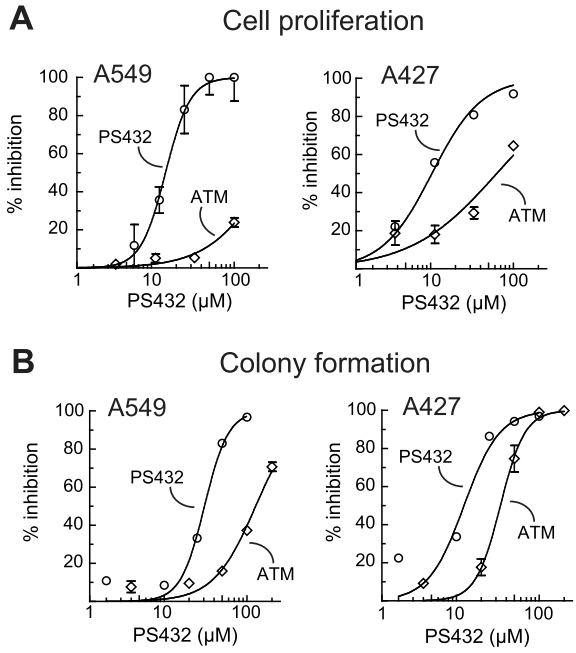

Figure 3. PS432 inhibits anchorage-dependent and -independent NSCLC cell growth. (A) A549 and A427 cells were exposed to various concentrations of PS432, ATM, or vehicle as a control. After 2 days, cell proliferation was assessed using the MTT assay, and the data are expressed as a percentage of cell growth inhibition with respect to vehicle-treated cells. Calculated IC_{50} values were 14.8 ± 4.2 μM for A549 and 10.4 ± 0.3 μM for A427. (B) Soft agar colony formation assay of A549 and A427 cells exposed to PS432, ATM, or vehicle. After cells had been treated for 7 days, their viability was assessed using alamar blue. The percentage of growth inhibition is presented. Results represent the mean ± SD of two or more experiments performed in triplicates. IC_{50} values were determined by fitting the data using nonlinear regression and were 30.7 ± 0.6 and 12.4 ± 0.6 μM for A549 and A427, respectively.

expression.[47] Therefore, we analyzed the expression of these genes in A549 cells after treatment with PS432 and found that PS432 inhibits their expression in a time- and concentration-dependent manner (SI Figure 5A,B). To gain additional insights into the cellular effects of this compound, we performed large scale gene expression and SILAC-based mass spectrometry (MS) analyses of A549 cells treated with 25 μM PS432 for various periods of time. Analysis of microarray data using $P < 0.01$ and a minimum 2-fold change in either direction from the gene's median value revealed 501 differentially expressed genes during a 24 h treatment period (SI Figure 6A). Hierarchical clustering of deregulated genes showed two major clusters containing 311 upregulated and 190 downregulated genes. Enrichment analysis of GO terms of differentially expressed genes indicated that PS432 had an effect in particular on the cell cycle, response to stress, changes in DNA conformation, and DNA replication (Figure 4A). Downregulated genes were involved in cell cycle-related biological processes, while upregulated genes were involved in tissue development, response to ER stress, and apoptosis among others (SI Figure 6B).

SILAC-based quantitative proteomic analysis of PS432-treated A549 cells showed that 145 proteins were differentially expressed over the 24 h treatment period. GO term enrichment analysis of the MS proteomic data set revealed also the effect of PS432 on cell cycle-related processes as well as macromolecule

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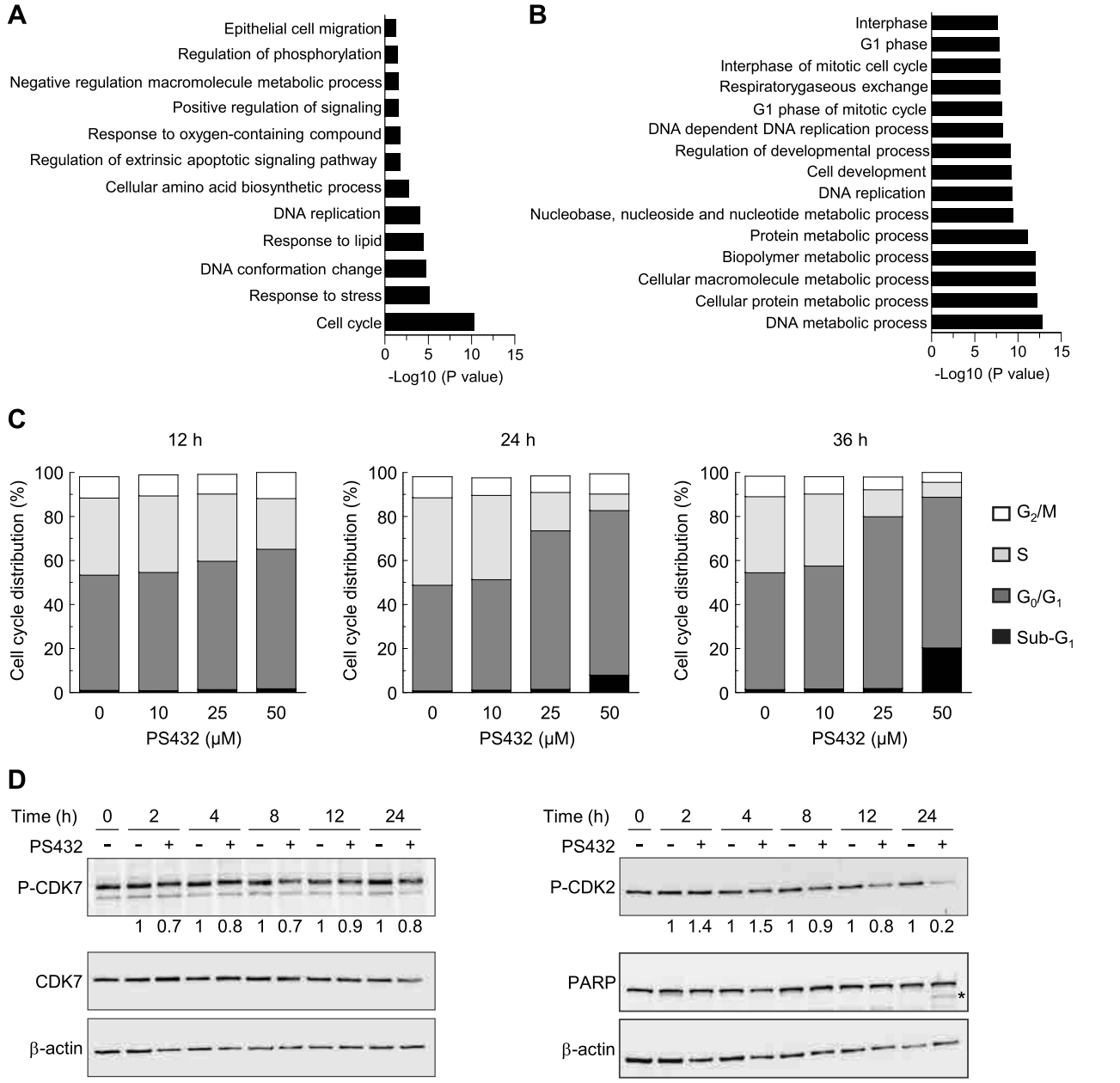

Figure 4. PS432 affects cell cycle-related processes. (A) Gene ontology terms of biological processes of genes that were differentially expressed in A549 lung cancer cells after treatment with PS432. (B) Gene ontology terms of biological processes of proteins that were differentially expressed in A549 lung cancer cells after treatment with PS432 determined by SILAC. (C) Cell cycle analysis of A549 lung cancer cells by flow cytometry. Cells were treated with different concentrations of PS432 for 12 (left), 24 (middle), or 36 h (right), and the DNA content was analyzed to reveal the cell distribution within the major phases of the cell cycle. PS432 induces arrest in the G_0/G_1 phase of the cell cycle. (D) A549 cells were enriched in the G_1 phase by serum starvation and then release in the presence or absence of 50 μM PS432. Whole cell lysates were obtained at the indicated times after treatment and subjected to Western blot analysis using anti-phospho-CDK7 (Thr170), anti-phospho-CDK2 (Thr160), or PARP antibodies. The signal was quantified, and the ratio of phosphorylated protein vs total protein or β-actin was calculated for P-CDK7 or P-CDK2, respectively. The indicated values below the blots represent the normalized signal intensities for P-CDK7 or P-CDK2 relative to the value of untreated control cells set at 1. Induction of apoptosis was detected by PARP cleavage. The 85 kDa form of cleaved PARP is denoted with an asterisk. β-Actin levels were used as a loading control.

metabolism (Figure 4B). Furthermore, flow cytometry analysis of the DNA content of A549 cells exposed to various concentrations of PS432 for different time periods revealed a time- and dose-dependent accumulation of cells in the G_0/G_1 phase of the cell cycle, accompanied by a decrease in the S phase fraction (Figure 4C). Treatment with 25 μM PS432 for 24 h resulted in an increase in the percentage of cells in the G_0/G_1 phase to 72% as compared with that of vehicle-treated control cells (48%). Exposure of A549 cells to PS432 for 36 h led to a further accumulation of cells in the G_0/G_1 phase of the cell cycle at a dose of 25 μM and to an increase in the sub-G_1 population, suggestive of apoptosis at the highest concentration of the compound (Figure 4C).

Because gene expression and proteomic analyses indicated that PS432 was affecting cell cycle-related processes and DNA content analysis demonstrated that PS432 induced G_0/G_1

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phase cell cycle arrest, we investigated the effect of PS432 (50 μM) on the phosphorylation of CDK7 and CDK2. CDK7 is a CDK-activating kinase that controls the activation of CDK2 during the cell cycle G_1/S transition.[48] CDK7 phosphorylation was partially inhibited by PS432 in synchronized A549 cells (Figure 4D). A similar decrease in the level of CDK7 phosphorylation has been described in neuroblastoma cells after inhibition of PKCι.[41] In addition, we found that after restimulation of A549 cells with serum, PS432 decreased the level of CDK2 activation that was clearly visible after 12 h (Figure 4D). This effect of PS432 on CDK2 activity is consistent with the previously reported delayed effect of CDK7 inhibition on T160 phosphorylation of CDK2 after mitogenic stimulation.[49] On the basis of these observations, we postulate that the cell cycle arrest caused by PS432 is mediated at least partially by the inhibition of CDK7 and CDK2. Together, the data indicated that PS432 inhibited cell proliferation of cancer cells by inducing cell cycle arrest in the G_0/G_1 phase followed by apoptotic cell death as revealed by cleavage of poly(ADP-ribose)polymerase (PARP) (Figure 4D and SI Figure 7).

PS432 in a Mouse Xenograft Model of Lung Cancer. The antitumor activity of PS432 was evaluated in a subcutaneous A549 xenograft tumor model. PS432 injected intraperitoneally (ip) at a dose of 2.5 mg/kg/day reduced the tumor volume by approximately 30% ($P < 0.01$) as compared to that of the control group at the end of the study (Figure 5A).

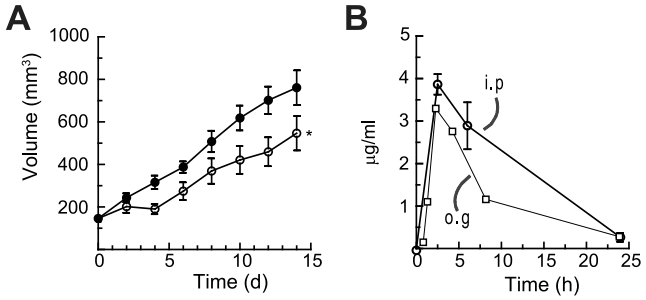

Figure 5. PS432 inhibits tumor growth in a xenograft model of lung cancer. (A) Tumor volume changes in A549 tumor-bearing mice treated ip with vehicle or PS432 at a dose of 2.5 mg/kg/day for 14 consecutive days. Data represent means ± the standard error of the mean. An asterisk indicates a significant difference ($P < 0.01$) between the mean tumor volume of the PS432- and vehicle-treated groups. (B) Concentrations of PS432 in the serum of A549 tumor-bearing mice after ip (2.5 mg/kg) or oral gavage (og, 10 mg/kg) administration of PS432.

This modest inhibitory effect correlates with the expected effect on the expression of genes regulated by PKCι[47] in the tumors treated with PS432 (SI Figure 5C). The results are consistent with the *in vivo* inhibition of PKCι, although we cannot exclude additional mechanisms of action for the observed effect of PS432 *in vivo*. Treatment with PS432 was tolerated well because there was no difference in the mean body weight of the control and treated mice throughout the experimental protocol (SI Figure 8A). Additionally, there was no difference in the weight of major organs, and their histopathological analysis confirmed the lack of toxicity (SI Figure 8B and data not shown). Serum concentrations were monitored for 24 h, with a c_{max} at 2.5 h (3.859 ± 0.243 μg/mL). Similar levels were obtained after oral administration of a 10 mg/kg dose (Figure 5B). The modest effect of PS432 *in vivo* could be due to the partial targeting of PKCι, because the maximal concentration of PS432 in serum is below the EC_{50} in cells in culture. Development of more potent compounds would eventually aid in our understanding of the mechanism of these allosteric compounds on tumor growth inhibition and if their action is mediated by modulation of aPKCs.

Synergy of PS432 in Combination Treatments. The therapeutic options for solid tumors remain disappointing. It is well established that growth of cancer cells is driven by mutations in several classes of genes. Thus, it can be suggested that tumors that rely on more than one signaling pathway would not be effectively treated by targeted drugs as single agents. Instead, cancer patients will likely benefit from rational combinatorial targeted therapies,[50] although the strategies for defining future personalized combination treatments remain undefined. Therefore, we investigated the potential use of the allosteric inhibitor PS432 in combination treatments with different drugs using a cross-titration approach. Most compounds tested, including topoisomerase inhibitors, DNA-damaging agents, and selective tyrosine kinase inhibitors, did not produce significant effects in combination with PS432. In contrast, we observed that the combination of PS432 with bortezomib or PI3 kinase inhibitors produced effects on the proliferation of A549, A427, DU145, and HCT116 cancer cells stronger than those of single-drug treatments (SI Figure 9). As shown in Figure 6, the concurrent treatment of A549 cells with PS432 and bortezomib (panel A) or BKM120 (panel B) induced synergistic effects that were dependent on the drug ratio with combination index (CI) values of <0.7 at high effect levels (ED ≥ 90).

It has been reported previously that compounds chemically similar to PS267 are classified as aggregators,[51] which may raise a doubt about the specificity of the PS267/PS432 family of compounds. Our biochemical and biophysical characterization of the compounds is performed in the presence of detergents that could mask their possible aggregation. Similarly, Dahlin and collaborators[52] call attention to promiscuous effects of a related scaffold. However, the authors found that aliphatic substitutions at any of the three positions of the 4-hydroxy-1,2-dihydropyrrol-5-one ring, as is the case with PS432 and the related compounds described herein, lower the risk of anomalous behavior.[52] In support of a specific binding to the target, PS267 selectively crystallizes in the PIF-pocket, the site to which it was designed to bind; also, PS432 primarily inhibits aPKCs without affecting related kinases.

We can speculate about the mechanism of synergy observed in cells based on the known roles of PI3 kinase and aPKCs. Growth factors stimulate cell proliferation and survival by triggering the activation of both PI3 kinase and small GTPase RAS signaling pathways. It is of interest that aPKCs have been extensively shown to play an important role downstream of K-RAS.[53,54] Therefore, under the combined inhibition of both kinases, PI3 kinase and aPKCs, growth factor signaling would be more efficiently inhibited. Also, constitutive PI3 kinase activity increased the level of expression and phosphorylation of PKCι,[55] suggesting the existence of another cross communication that could lead to synergistic effects after concurrent treatment with inhibitors targeting both kinases.

It is of interest to note that allosteric compounds induce a shift in the population dynamics of the kinase in manners that are yet to be described in detail. A full understanding of the molecular mechanism underlying protein structure and dynamics would facilitate the future rational design of allosteric compounds. Without a full understanding of the dynamics of

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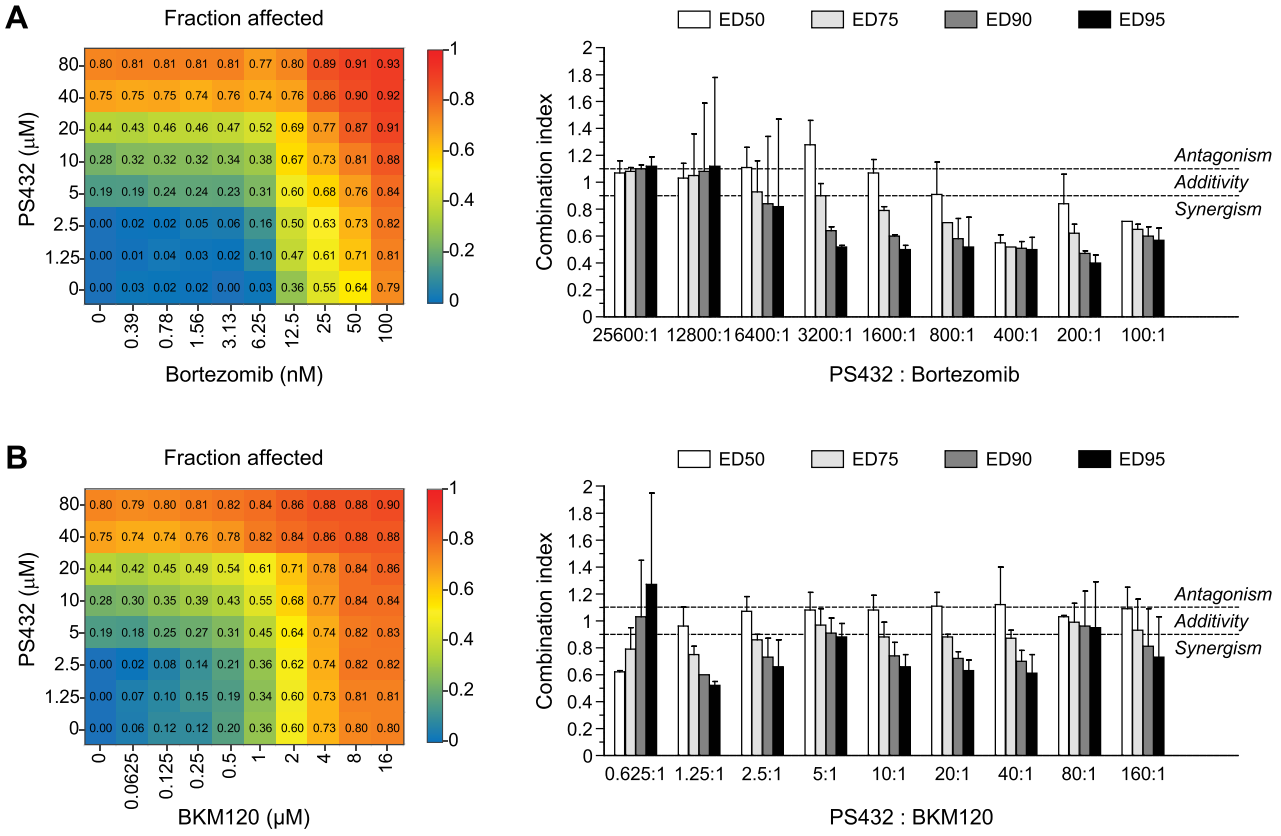

Figure 6. Concurrent treatment with PS432 and proteasome or PI3 kinase inhibitors exhibits a synergistic inhibitory effect on A549 cell proliferation. (A) Heat map (left) of two-way titration experiments for the calculation of the combination index (CI) of PS432 and bortezomib. Inset values indicate the fraction affected. CI values (right) at the indicated PS432:Bortezomib concentration ratios at different effect levels (ED_{50}, ED_{75}, ED_{90}, and ED_{95}) calculated using the CompuSyn software. Bars represent the mean ± SD of two different experiments performed in triplicate. Dashed horizontal lines indicate antagonism (CI > 1.1), additivity (0.9 < CI < 1.1), and synergy (CI < 0.9). (B) Same as panel A except for the combination of PS432 and BKM120.

the system, compounds optimized to bind to the PIF-pocket could turn out to be allosteric activators, allosteric inhibitors, or compounds inducing only partial effects. The recent molecular dynamics characterization of the bidirectional allosteric modulation of the conformation of the PIF-pocket and the ATP-binding site by small compounds binding to either site[33] suggests strategies for the future full rational design of drugs for protein kinases with a desired allosteric effect. In the meantime, our work provides evidence of the success of rational optimization of allosteric inhibitory compounds for aPKCs by focusing on the occupation of the deep tunnel, a feature that we previously described to affect the active site of aPKCs inhibiting its activity.

METHODS

Synthesis and Characterization of Ethyl 4,5-Dioxo-pyrrolidine-3-carboxylate Derivatives. All ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded on Bruker AM 250 (250 MHz) or AMX 400 (400 MHz) spectrometers. Matrix-assisted laser desorption ionization mass spectra were recorded on a Fisons VG Tofspec spectrometer and ESI mass spectra on a Fisons VG Platform II spectrometer. The purity of the compounds was determined by high-performance liquid chromatography and mass spectrometry and was higher than 95%. A solution of the corresponding amine (1 equiv) and aldehyde (1 equiv) in a mixture of ethanol and acetic acid (4:1) was refluxed for 30 min, and sodium diethyl oxaloacetate (1 equiv) was added. The mixture was refluxed for 16 h (conventional method) or 4 h (100 °C, power *P* of 150W; microwave-supported synthesis) using a Discover microwave (CEM Corp.). After dilution with diethyl ether, the precipitate was filtered, washed with diethyl ether, and dried under reduced pressure. The crude product was purified via column chromatography or recrystallization in mixture of methanol and diethyl ether. Besides an increase in potency, the pharmaceutical properties of PS432 require optimization, including a higher water solubility (PS432 calculated log *P* = 4.9). A full description of the SAR of a larger number of compounds from the series will be published elsewhere (D. Odadzic et al., manuscript to be submitted for publication).

Structural Studies. PDK1-chimeric protein was prepared as described previously.[21] In brief, the construct is based on the catalytic domain of PDK1 ($dmPDK1_{50-359}$)[8,9,12−14] and comprises eight mutations at the PIF-pocket to mimic the pocket of PKC*ι*. The chimeric protein was expressed in SF9 insect cells as a fusion to a His tag, which was then cleaved with TEV protease during the purification procedure. X-ray crystallographic studies are described in the Supporting Information. The coordinates of the dmPDK1−PKC*ι* chimera in complex with PS267 have been deposited in the Protein Data Bank as entry 5MRD.

Biochemical Assays. Recombinant proteins were expressed and purified, and their activity was measured *in vitro* as described previously.[19,22] Interaction assays were performed using the Alpha-Screen (amplified luminescent proximity homogeneous assay) technology, as described previously.[19,21] The bead-based assay allows the detection of biomolecular interactions and the effect of small compounds. Briefly, the Alpha-Screen assay was performed in a final volume of 25 μL in white 384-well microtiter plates (Greiner Bio-One) with His-PKC*ι*-CD (40 nM) and biotin-pseudosubstrate (80 nM) or His-PKC*ι*-CD (15 nM) and biotin-ROCKtide (100 nM) in a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.01%

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(v/v) Tween 20, 0.1% (w/v) BSA, 2 mM dithiothreitol (for the PKCι–Rocktide interaction) or 5 mM dithiothreitol (for the PKCι–pseudosubstrate interaction), and the corresponding concentration of the compound. Five microliters of beads at a concentration of 20 μg/mL was added to the mixture (nickel chelate-coated acceptor beads and streptavidin-coated donor beads), and after incubation for 90 min, Alpha-Screen counts were obtained in an EnVision Multiplate reader.

Cellular Assays. A detailed description of cellular assays can be found in the Supporting Information.

***In Vivo* Experiments.** Six- to eight-week-old female HsdCpb:NMRI-Foxn1nu mice (Harlan) were inoculated subcutaneously with 5×10^6 A549 cells in a total volume of 0.1 mL of serum-free medium containing 25% Matrigel (BD Biosciences). When tumors reached an average volume of approximately 150 mm^3, mice were treated with PS432 at a dose of 2.5 mg/kg/day or vehicle (12% DMSO in PBS) administered ip. Tumor growth was monitored every 2 days as described previously.[56] For the analysis of PS432 concentrations in serum, blood was drawn from the mice at the indicated times after the last ip injection, and PS432 was then quantified from the sera by mass spectrometry (Pharmacelsus GmbH). At necropsy, major organs were grossly examined, removed, and weighed. All animal experiments were performed in accordance with the German regulations for animal care, and the experimental protocols were approved by the Animal Welfare Commission of the State of Hessen and the University of Frankfurt.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00827.

Supplementary methods, Tables S1–S3, and SI Figures 1–9 (PDF)

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Author Contributions

J.M.A. was responsible for cellular and animal experiments and was supported by M.K. and P.M. during the cellular experiments and M.K., C.S., and V.K. during animal work. J.M.A. and T.O. performed and analyzed the SILAC experiments. D.P.-F. performed yeast-based experiments. The crystallography work was performed by J.O.S. E.S. did Alpha-Screen and biochemical experiments, which were supervised by S.N. and R.M.B. S.Z., A.P., and H.S. analyzed data and discussed results. W.F. first synthesized PS212 and numerous derivatives, including PS432, in the laboratory of Matthias Engel. D.O. analyzed data, synthesized and characterized compounds. R.M.B. directed the overall research project. The manuscript was written by R.M.B. and J.M.A. with the support of M.K., J.O.S., and D.O.

Notes

The authors declare no competing financial interest.

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