Novel p21-Activated Kinase 4 (PAK4) Allosteric Modulators Overcome Drug Resistance and Stemness in Pancreatic Ductal Adenocarcinoma

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Abstract

The p21-activated kinase 4 (PAK4) is a key downstream effector of the Rho family GTPases and is found to be overexpressed in pancreatic ductal adenocarcinoma (PDAC) cells but not in normal human pancreatic ductal epithelia (HPDE). Gene copy number amplification studies in PDAC patient cohorts confirmed PAK4 amplification making it an attractive therapeutic target in PDAC. We investigated the antitumor activity of novel PAK4 allosteric modulators (PAM) on a panel of PDAC cell lines and chemotherapy-resistant flow-sorted PDAC cancer stem cells (CSC). The toxicity and efficacy of PAMs were evaluated in multiple subcutaneous mouse models of PDAC. PAMs (KPT-7523, KPT-7189, KPT-8752, KPT-9307, and KPT-9274) show antiproliferative activity in vitro against different PDAC cell lines while sparing normal HPDE. Cell growth inhibition was concurrent with apoptosis induction and suppression of colony formation in PDAC. PAMs inhibited proliferation and antiapoptotic signals downstream of PAK4. Co-immunoprecipitation experiments showed disruption of PAK4 complexes containing vimentin. PAMs disrupted CSC spheroid formation through suppression of PAK4. Moreover, PAMs synergize with gemcitabine and oxaliplatin in vitro. KPT-9274, currently in a phase I clinical trial (clinicaltrials.gov; NCT02702492), possesses desirable pharmacokinetic properties and is well tolerated in mice with the absence of any signs of toxicity when 200 mg/kg daily is administered either intravenously or orally. KPT-9274 as a single agent showed remarkable antitumor activity in subcutaneous xenograft models of PDAC cell lines and CSCs. These proof-of-concept studies demonstrated the antiproliferative effects of novel PAMs in PDAC and warrant further clinical investigations.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a deadly malignancy and the fourth leading cause of cancer-related deaths in the United States (1). Oncogenic Ras (Kras) is the most prevalent somatic aberration (≥95% mutations) in PDAC (2). Because Kras is a GTPase, the protein acts as a molecular switch recruiting and activating a repertoire of effectors necessary for cell proliferation, desmoplasia, maintenance of tumor heterogeneity (cancer stem–like cells or CSCs), tumor aggressiveness, and metastasis (3). Even though there is a consensus regarding the importance of oncogenic Kras in PDAC, the absence of a typical druggable binding pocket in its structure has resulted in Kras remaining an elusive target for several decades (4). To overcome this challenge, identification of novel target sites in the Ras structure itself, in critical direct interactors, or in downstream effectors is urgently needed (5).

The p21-activated kinase (PAK) family members are key effectors of the Rho family of GTPases downstream of Ras, which act as regulatory proteins controlling critical cellular processes such as cell motility, proliferation, and survival (6). One of the family members, PAK4, is a key effector of Cdc42 (cell division control protein 42 homolog) and acts as a critical mediator of Ras-driven activation (7). In earlier studies, copy number alteration analysis showed amplification of PAK4 in patients with PDAC (8). High-resolution genomic and expression profiling in a large number of cellular and patient models have revealed putative amplifications of the PAK4 gene that has been linked to cell migration, cell adhesion, and anchorage-independent growth in PDAC (9). Therefore, PAK4 is an attractive small-molecule target in the elusive Ras pathway. In addition, PAK4 inhibition is proposed to overcome gemcitabine resistance by suppressing Kras-mediating proliferation of PDAC.

Earlier attempts to target PAK4 resulted in the development of an ATP-competitive Type I kinase inhibitor, PF-03758309 (tested in non-pancreatic models; ref. 10). This compound was discontinued after a single clinical trial due to undesirable pharmacokinetic characteristics (low human bioavailability) and lack of efficacy. Although there are several PAK4 inhibitors reported in the literature (11), none made it to the clinic. There are no studies reported that investigate the role of PAK4 as a therapy in resistant PDAC, and thus there is a void in our understanding of the impact of PAK4 inhibition in this deadly disease. Here, we show that...
inhibition of PAK4 by novel allosteric modulators (KPT-9274 and analogs) can suppress PDAC proliferation, reverse cancer stemness, and promote synergism with chemotherapeutic agents, gemcitabine and oxaliplatin, both in vitro and in vivo.

Materials and Methods

Cell lines and culture conditions and research reagents

PDAC cells (MiaPaCa-2, HPAC, and Panc1) were purchased from ATCC. Colo-357 and L3.6pl cells were provided by Dr. Paul J Chiao (MD Anderson, Houston, TX). Human pancreatic ductal epithelia (HPDE) cells were generously provided by Dr. Mien-Sound Tsao (Department of Pathology, Montreal General Hospital, Quebec, Canada). The cells were grown in DMEM culture medium (Invitrogen) complemented with 10% FBS (Cambrex), 100 U/mL penicillin/streptomycin (Invitrogen), and 2 mmol/L l-glutamine (Invitrogen). Cell lines used in this study were obtained in 2009. MiaPaCa-2, HPAC, and Panc1 have been authenticated yearly and recently on July 14, 2016. The method used for testing was STR profiling using the PowerPlex 16 System at Genetica. MiaPaCa-2 CSCs were developed by flow sorting authenticated MiaPaCa-2 cells for CD44 CD133 and EpCAM. The cells were grown in DMEM/F12 medium supplemented with B-27 and N-2 (Invitrogen) in ultra-low attachment 6-well plates (Corning). These cells sustained in long-term culture conditions and show stem cell characteristics with mesenchymal markers (12, 13). PAK4 allosteric modulators (PAM) were provided by Karyopharm Therapeutics. Primary antibodies for PAK4, p-PAK4, GEFH1, cyclin D1, ERK1/2, vimentin, and p65 were purchased from Cell Signaling. All the secondary antibodies were obtained from Sigma.

Identification of PAK4 as the target PAMs utilizing stable isotope labeling of cells

PAMs (KPT-7523, KPT-9274, KPT-7189, and KPT-9307 or their related analogs) were obtained from Karyopharm Therapeutics (14). Briefly, PAMs were immobilized on a resin through a PEG linker. MS751 cells (cervical cancer) were labeled with heavy/light arginine and lysine for at least 6 doublings. MS751 cells are sensitive to KPT-7523 in vitro (MTT at 72 hours IC_{50} = 30 nmol/L). Labeled cells were lysed in modified RIPA buffer and treated with DMSO or excess KPT-7523 for 2 hours. The pretreated lysates were then incubated and rotated with KPT-7523-resin overnight at 4°C to pull-down interacting proteins. The next day, light and heavy resin were washed and then mixed in equal proportions. The resin samples were boiled and the purified proteins were run on SDS-PAGE. Proteins were cut from the gel, trypsin-digested, and then identified through mass spectrometry. KPT-7523–interacting proteins were identified as those having a heavy-light ratio with more than 2-fold enrichment. PAK4 was identified as the strongest interactor with about 32-fold enrichment across 4 different replicates. A similar SILAC experiment in U2OS cells (MTT at 72 hours IC_{50} = 20 nmol/L) confirmed these results. Follow-up biophysical assays (isothermal titration calorimetry, surface plasmon resonance, and X-ray crystallography) confirmed the interaction between PAK4 and KPT-7523. Using exogenous, endogenous, and purified protein from cells, KPT-7523 showed specific interaction to PAK4 and not to PAK5 or PAK6. There was no interaction with any of the group I PAK proteins. The interaction between PAK4 and KPT-7523, however, did not impact the kinase activity of PAK4. The interaction disrupts steady-state levels of PAK4 in cell lines and reduces overall PAK4 activity. Ultimately, PAK4 downstream signaling is modulated by treatment with PAMs.

Cell growth inhibition by MTT

PDAC cells were seeded at a density of 5 × 10^4 cells per well in 96-well microtiter culture plates. After overnight incubation, medium was removed and replaced with fresh medium containing PAMs at indicated concentrations (0–5,000 nmol/L) diluted from a 1 mmol/L stock. After 72 hours of incubation, MTT assay was performed by adding 20 μL of MTT (Sigma) solution (5 mg/mL in PBS) to each well and incubated further for 2 hours. Upon termination, the supernatant was aspirated, and the MTT formazan formed by metabolically viable cells was dissolved in 100 μL of isopropanol. The plates were gently rocked for 30 minutes on a gyrotrary shaker, and absorbance was measured at 595 nm using a plate reader (TECAN).

Clonogenic assay

Fifty thousand cells were seeded in 6-well plates and allowed to grow for 24 hours. Once attached, the cells were exposed to increasing concentrations of different PAMs (either alone or in combination with gemcitabine and oxaliplatin) for 72 hours. At the end of the treatment period, 1,000 cells were taken from each reaction well and reseded in 100-mm petri dish and allowed to grow for 2 weeks at 37°C in a 5% CO₂/5% O₂/90% N₂ incubator. Colonies were stained with 2% crystal violet, counted, and quantitated.

Sphere formation/disintegration assay

Single-cell suspensions of flow-sorted MiaPaCa-2 CSC spheroids were plated on ultra-low adherent wells of 6-well plates (Corning) at 1,000 cells per well in sphere formation medium (1:1 DMEM/F12 medium supplemented with B-27 and N-2; Invitrogen). After 7 days, the spheres were collected by centrifugation (300 × g, 5 minutes) and counted. The proportion of sphere-generating cells was calculated by dividing the number of spheres by the number of cells seeded. The sphere formation assay of secondary spheres was conducted by using primary spheres. Briefly, primary spheres were harvested and incubated with Accutase (Sigma) at 37°C for 5 to 10 minutes. Sphere disintegration assay was performed by growing 1,000 cells per well on ultra-low adherent wells of 6-well plate in the sphere formation medium and incubated with PAMs (increasing concentrations, 0–1,000 nmol/L) for a total of 14 days following 2 days a week of drug treatment, and the cells were harvested as described previously (12). The spheres were collected by centrifugation and counted under a microscope as described above.

Quantification of apoptosis by Annexin V/FITC assay

Cell apoptosis was detected using Annexin V/FITC (Biovision) according to the manufacturer’s protocol. PDAC cells were seeded at a density of 50,000 cells per well in 6-well plates in 5 mL of corresponding media. Twenty-four hours after seeding, the cells were exposed to PAMs at different concentrations for 72 hours. At the end of treatment period, cells were trypsinized and equal numbers were stained with Annexin V and propidium iodide (PI). The stained cells were analyzed using a Becton Dickinson flow cytometer at the Karmanos Cancer Institute Flow cytometry core.
Cell-cycle analysis

Cells were plated out at a density of 50,000 per well in regular DMEM (with 10% FBS) in 6-well plates. The cells were then serum starved for 24 hours. The media were replaced with regular media containing PAMs at the indicated concentrations for 72 hours. The cells were then harvested by trypsinization, washed once in cold PBS, and resuspended in 4 mL of assay buffer (Cayman Chemicals). The suspended cells were fixed by addition of 1 mL of fixative solution and incubated at 4°C overnight. Cells were then washed with PBS and incubated with 50 µg/mL PI and 100 µg/mL ribonuclease A for 30 minutes at 37°C according to the manufacturer’s protocol (Cayman Chemicals). Cells were analyzed on the Fortessa flow cytometer (BD), and results were analyzed using the FlowJo Software. Experiments were done in duplicate and repeated 2 times.

Wound-healing scratch assay

PDAC cells were grown in triplicate at a density of 100,000 cells per well in 6-well plates. The cells were seeded in the 6-well plates in 3 mL of regular DMEM in each well. When the confluence of the cells grew to about 95%, the wells were then scratched with a 200-µL pipette tip followed by incubation with KPT-7523 (500 nmol/L). The wound-healing capacity was assessed and photographed with a camera-equipped inverted microscope after 72 hours of incubation.

Western blot analysis

A total of 1 × 10⁶ PDAC cells were grown in 100-mm petri dishes overnight to about 50% confluence. Next day, cells were exposed to indicated concentrations of PAMs for 72 hours, followed by extraction of protein for Western blot analysis. Preparation of cellular lysates, protein concentration determination, and SDS-PAGE analysis were done as described previously (15).

siRNA and transfections

To study the effect of PAK4 silencing on activity of PAMs, we utilized siRNA silencing technology. PAK4 siRNA and control siRNA were obtained from Santa Cruz Biotechnology. Cells were transfected with either control siRNA or PAK4 siRNA for 24 hours using Lipofectamine 2000 (Invitrogen) for 2 cell passages according to the manufacturer’s protocol. siRNA silencing was verified using RT-PCR. All procedures have been standardized and published previously (16). The primers used for PAK4 were Forward primer: 5’-ATG TTT TGG AAG AAG AAG C-3’ and Reverse primer: 5’-TCA TCT GGT GCC GTT CTG GCC GGC-3’. After the siRNA treatment period, cells were further treated with PAMs (at IC₅₀ concentrations) in 96-well plates for MTT and 6-well plates for Annexin V/FITC assays, respectively.

Immunofluorescence and confocal microscopy

PDAC cells and MiaPaCa-2 CSCs were grown on glass chamber slides and exposed to PAMs at indicated concentrations for 72 hours (or otherwise stated). At the end of the treatment, the cells were fixed with 10% paraformaldehyde for 20 minutes. The fixed slides were blocked in TBSB and probed with primary and secondary antibodies according to our previously published methods (16). The slides were dried and mounting medium was added to it and covered with a coverslip and were analyzed under an inverted fluorescent microscope. A total of 2 independent experiments were performed.

Animal xenograft studies

All in vivo studies were conducted under Animal Investigation Committee-approved protocol. Four-week-old female ICR-SCID mice (Taconic farms) were adapted to animal housing and xenografts were developed as described earlier (15). To test the efficacy of PAM KPT-9274, bilateral fragments of the L3.6pl or AsPC-1 xenograft were implanted subcutaneously into naïve, similarly adapted mice. Five days later, both L3.6pl and AsPC-1 cells developed into palpable tumors (≈50 mg), and these tumor-bearing animals were randomly assigned to different cohorts and treated with either diluents (control group) or 140 mg/kg of KPT-9274 intravenously 5 times per week, for 4 weeks. All mice were followed for measurement of subcutaneous tumor weight and observed for changes in body weight and any side effects.

Statistical analysis

Wherever appropriate, the data were subjected to a paired 2-tailed Student’s t-test. P < 0.05 was considered statistically significant.

Results

PAMs suppress PDAC proliferation

A series of novel orally bioavailable small molecules, identified by Karyopharm Therapeutics, interact with the PAK4 protein but do not inhibit its kinase activity. Instead these PAMs affect steady-state phosphorylation and total PAK4 protein levels in cancer cells. The interaction with PAK4 was first discovered using SILAC and a resin tagged compound, KPT-7523, as described in Materials and Methods (14, 17). Figure 1A shows the structure of PAM analogues investigated in this study. PAMs show antitumor activity in both solid and hematologic cell lines in low nanomolar EC₅₀ concentrations (Fig. 1B, Supplementary Fig. S1; refs. 14, 17). MTT assays were used to verify the antitumor activity of PAMs against PDAC cells in vitro. As seen in Figure 1B, most PAM analogs show potent dose-dependent anticancer activity against a spectrum of PDAC cell lines (EC₅₀ values: ~500 nmol/L in PDAC cells) without affecting normal HPDE cells (EC₅₀ > 5 µmol/L). These results clearly show that inhibition of PAK4 can suppress PDAC cell proliferation and can be used as a selective therapeutic strategy against oncogenic Kras-driven pancreatic tumor cells.

PAMs induce apoptosis, cell-cycle arrest and suppress migration in a PDAC cell selective manner

To determine whether PAMs mediated apoptosis, Annexin V/FITC assay was performed. Figure 2A and 2B show results of Annexin V/FITC assay comparing PAM activity in PDAC cells. PAMs induce substantial apoptosis in PDAC at 500 and 1,000 nmol/L treatment for 96 hours. PAMs were also effective in inducing apoptosis in a panel of additional PDAC cells lines (Supplementary Fig. S2). Molecular analysis of KPT-9274-treated MiaPaCa-2 PDAC cells demonstrated significant PARP cleavage (Fig. 2C). Most significantly, PAMs did not induce apoptosis in normal HPDE cells (Fig. 2D).

Earlier work from multiple independent research groups has shown that knocking down PAK4 by RNA interference (RNAi) suppressed the proliferation of PDAC cells (8, 18). On the basis of these published findings, we first explored whether PAK4 silencing has similar impact on our PDAC cell lines. Indeed, siRNA silencing of PAK4 resulted in statistically significant reduction of proliferative potential of MiaPaCa-2 and L3.6pl (Supplementary Fig. S3A and S3B shows MTT assay and Supplementary Fig. S3C...
and S3D showing efficient PAK4 knockdown upon siRNA treatment by RT-PCR). These observations demonstrated the critical need (oncogenic addiction) of PAK4 in subsistence of PDAC. We also investigated whether PAK4 suppression by siRNA could enhance the activity of PAMs. PAM-mediated apoptosis in MiaPaCa-2 and L3.6pl is enhanced by PAK4 knockdown in these cell lines (Supplementary Fig. S3E). It should be noted that siRNA only partially suppresses PAK4 protein in these cells indicating that PAMs require lesser dose to induce apoptosis (Supplementary Fig. S3C and S3D).

Studies have shown that siRNA silencing of PAK4 results in the induction of cell-cycle arrest in PDAC cells (18). Therefore, we investigated the impact of PAMs on different phases of cell cycle using flow cytometric analysis. When compared with control, treatment with PAMs for 72 hours resulted in consistent G2 phase arrest (~10% change) in PDAC cells (Supplementary Fig. S4A and S4B) but not in normal HPDE cells (Supplementary Fig. S4C). These results are consistent with existing siRNA work in the literature and demonstrate that PAK4 inhibition by PAMs interfere with cell-cycle regulatory pathways. The Rho GTPases and their downstream effectors which include PAK proteins play an important role in cellular migration (19). Therefore, we sought to investigate the impact of PAMs on PDAC cellular migration using a standard scratch assay. When compared with vehicle control, treatment with 500 nmol/L KPT-7523 for 48 hours resulted in substantial inhibition of scratch closure in confluent MiaPaCa-2 or L3.6pl cells (Supplementary Fig. S5A and S5B). These results support the link between PAK proteins and cellular migration of cancer cells.

**PAMs suppress PAK4-related signaling**

Two recent publications have shown the specificity of PAMs toward PAK4 in distinct tumor models (14, 17). We evaluated the impact of PAMs on PAK4 and downstream signaling using Western blotting. Exposure of MiaPaCa-2 cells to KPT-7189 (0–2 µmol/L) or MiaPaCa-2 and L3.6pl cells to KPT-9307 (0–200 nmol/L) for 72 hours resulted in reduction of total PAK4, phospho-S474-PAK4 (p-PAK4) and cyclin D1 (Fig. 3A and B). Decrease in other major downstream signaling molecules such as ERK1/2, GEF-H1, phospho-ERK1/2, and YAP was not that drastic (data not shown). KPT-9274 and KPT-9307 reduced p-PAK4 level without affecting p-PAK2 demonstrating specificity.
toward PAK4 (Fig. 3C). It is important to note that the PDAC cell lines used in this study do not express all PAK isoforms. Therefore, to exclude the role of group I PAKs in the activity of PAMs, we utilized DU-145 prostate cancer cell line that expresses PAK1, PAK2, and PAK3 as well as group II PAKs. As shown in Supplementary Fig. S6, treatment of DU-145 with a sub-IC50 amount of KPT-9274 resulted in selective inhibition of p-PAK4 sparing other group I PAKs.

Next, we used a co-immunoprecipitation assay to determine whether PAMs could disrupt the interaction between PAK4 and its binding partners. In Fig. 3D, we demonstrated that KPT-9274 treatment resulted in the disruption of PAK4–vimentin interaction when compared with control treatment. These studies demonstrated that PAM-mediated suppression of cellular proliferation, apoptosis, and cell-cycle arrest were due, in part, to the suppression of PAK4 signaling.

Overcoming stemness through PAK4 inhibition

In previous studies, we have shown that flow sorting PDAC cells for stem-like markers (CD33+ CD44+ EpCAM+ or CSCs) demonstrated marked resistance to standard-of-care chemotherapeutics such as gemcitabine and oxaliplatin (12). These flow-sorted cells harbor mesenchymal markers and have a propensity to form spheroids in long-term culture conditions. Highlighting the critical role of PAK4 in the PDAC stem cell biology, we observed marked enhancement in PAK4 mRNA in gemcitabine-resistant MiaPaCa-2 (MiaPaCa-2-GR) as well as MiaPaCa-2 CSCs (Fig. 4A). RNAi of PAK4 in these CSCs suppressed their propensity to form...
spheroids in a statistically significant manner (Fig. 4B). PAM treatment (twice a week for 2 weeks) was found to disrupt spheroids in a dose-dependent manner (Fig. 4C). PAMs could induce apoptosis (Supplementary Fig. S7A and S7B) in CSCs and downregulate epithelial–mesenchymal transition (EMT) markers such as CD24, CD44, EpCAM (Fig. 4D), and Snail (Supplementary Fig. S7C). Collectively, these data show that PAMs can suppress drug-resistant subpopulation of PDAC CSCs with mesenchymal traits.

PAMs synergize with gemcitabine and oxaliplatin

Given that PAK4 is overexpressed in gemcitabine-resistant PDAC cells, we evaluated whether PAMs can synergize with 2 commonly used standard-of-care therapies in pancreatic cancer treatment: gemcitabine and oxaliplatin. KPT-9274 synergized with gemcitabine and oxaliplatin in 2 PDAC cell lines in vitro (Fig. 5A and B). Isobologram analysis showed that the combination index of KPT-9274 with either compound was synergistic (CI < 1). To further demonstrate the synergy, we performed apoptosis analysis on the KPT-9274 combination–treated cells. The combination of KPT-9274 with gemcitabine or oxaliplatin led to statistically significant enhancement in apoptosis when compared with either single agent alone (Fig. 5C and D). Using a clonogenic assay, we observed inhibition of colony formation after KPT-9274 combination treatment when compared with single agents alone in both cell lines tested (Fig. 5E and F). We demonstrated synergy at the molecular level using RT-PCR analysis which demonstrated downregulation of PAK4 mRNA in the combination treatment when compared with single agents (P < 0.01) in both PDAC cell lines (Supplementary Fig. S8A–S8D). Collectively, the results
provide strong preclinical rationale for the application of PAMs in combination with gemcitabine and oxaliplatin for the treatment of therapy-resistant PDAC.

KPT-9274 shows antitumor activity as a single agent and in combination with gemcitabine in preclinical animal tumor models. Prior to human clinical evaluation, the impact of any new anticancer agent needs to be validated in suitable animal tumor models. Therefore, we evaluated the antitumor activity of the PAM analog, KPT-9274, in xenografts of PDAC grown subcutaneously in SCID mice. As can be seen in Figure 6A, intravenous administration of KPT-9274 at 140 mg/kg for 5 days per week completely eliminated the L3.6pl PDAC tumors. We did not see any outward signs of toxicity, body weight loss (Fig. 6B), or tumor rebounding (Fig. 6C showing lack of tumors in treated mice) in the treated group before termination of the experiment. To verify whether PAMs could regress established PDAC tumors, we exposed the control group mice (with mean tumor volume of 1,500 mg) at day 21 of the experiment to KPT-9274 (140 mg/kg i.v.) twice a week for 2 weeks. The spheroids were counted under a microscope and photographed. Excised tumors were examined by hematoxylin and eosin (H&E) staining and confirmed tumor cells were still present (Fig. 6A, inset).

The L3.6pl studies were supported by another xenograft study harboring AsPC-1 tumors. In this model, treatment with KPT-9274 also demonstrated statistically significant inhibition of tumor growth (Supplementary Fig. S9A; \( P < 0.05 \)). We used sub-MTD (maximum tolerated dose) oral doses of PAMs to evaluate synergy with gemcitabine. In line with the in vitro results, the combination studies with gemcitabine (given at 50 mg/kg i.v.) showed marginal and statistically enhanced (\( P < 0.05 \)) antitumor activity (Supplementary Fig. S9B). Collectively, these findings clearly build the strong rationale for PAMs as potential therapeutics against PDAC that warrant further clinical investigations.

KPT-9274 shows antitumor activity as a single agent and in combination with gemcitabine in preclinical animal tumor models of PDAC

Prior to human clinical evaluation, the impact of any new anticancer agent needs to be validated in suitable animal tumor models. Therefore, we evaluated the antitumor activity of the PAM analog, KPT-9274, in xenografts of PDAC grown subcutaneously in SCID mice. As can be seen in Figure 6A, intravenous administration of KPT-9274 at 140 mg/kg for 5 days per week completely eliminated the L3.6pl PDAC tumors. We did not see any outward signs of toxicity, body weight loss (Fig. 6B), or tumor rebounding (Fig. 6C showing lack of tumors in treated mice) in the treated group before termination of the experiment. To verify whether PAMs could regress established PDAC tumors, we exposed the control group mice (with mean tumor volume of 1,500 mg) at day 21 of the experiment to KPT-9274 (140 mg/kg i.v.) twice a week for 2 weeks. The spheroids were counted under a microscope and photographed. Excised tumors were examined by hematoxylin and eosin (H&E) staining and confirmed tumor cells were still present (Fig. 6A, inset).

Figure 4. Impact of PAMs on PDAC CSCs. A, MiaPaCa-2 cells flow sorted for CD44+CD133+EpCAM+ according to our previously published procedure (12). In addition, MiaPaCa-2 cells were grown for extended period of time in gemcitabine (100 nmol/L) to develop resistance cell (MiaPaCa-2 GR). RNA isolated from CSCs or MiaPaCa-2 GR were evaluated using RT-PCR for basal expression of PAK4. B, The sorted cells were exposed to either control siRNA or PAK4 siRNA according to established procedures (15). The spheroid formation in PAK4 siRNA exposed CSCs was evaluated over 2 weeks, and the cell spheroids were counted and photographed under an inverted microscope (*, \( P < 0.01 \) between control and PAK4 siRNA treatment groups). C, In a separate experiment, the flow-sorted CSCs were grown in ultra-low adherent 6-well plates and in spheroid-forming media DMEM/F-12 with N-2 and B-27 supplement (Invitrogen) and exposed to increasing concentrations of PAMs (0-1,000 nmol/L) twice a week for 2 weeks. The spheroids were counted under a microscope and photographed. D, MiaPaCa-2 CSCs grown in regular media were exposed to different PAMs (5 μmol/L) for 72 hours. At the end of the treatment period, RNA was isolated and RT-PCR was performed as described in Materials and Methods. Note: downregulation in stemness markers CD24, CD44, and EpCAM.

PAM suppresses highly resistant PDAC CSC–derived tumors

Given that PAMs are effective against CD44+/CD133+/EpCAM+ (triple marker–positive cells), PDAC CSCs in vitro...
led us to evaluate their activity against the same model in vivo. These CSCs are highly resistant to conventional chemotherapeutics, as they possess stemness markers. Results of Figure 7A show that CSCs tumors do not respond to gemcitabine (given at 50 mg/kg intraperitoneally) or nab-paclitaxel (given at 30 mg/kg i.v. once a week for 2 weeks). Data summarized in Figure 7B show that exposure of the chemotherapy-resistant xenograft to KPT-9274 (140 mg/kg) resulted in almost complete suppression of CSC tumors. More striking were the results from the control group treated with KPT-9274 at a later time point (when tumors were reached \( C_24 \)). Treatment of these large-sized control tumors with KPT-9274 led to statistically significant reduction in tumor size. Marker analysis of the control residual tumor demonstrated consistent overexpression of CSC markers confirming that these tumors contained stem cells (data not shown). Collectively, these multiple lines of in vitro and in vivo evidence demonstrate the potential to use KPT-9274 as an antitumor agent and warrants further preclinical and clinical evaluation for the treatment of therapy-resistant PDAC.

**Discussion**

In this report, we demonstrate antitumor activity of novel PAMs as single agent or in combination with chemotherapies in PDAC, highly resistant CSCs, and xenograft tumor models. Despite intensive research in the last decade, there has been no significant progress in either the identification of early diagnostic markers or the development of novel therapies for this deadly disease (20). Deaths from cancers of the pancreas are projected to overtake a majority of other cancers by the year 2030 (21). While the oncogenic Kras signaling has been recognized to play a central role in the aberrant biology of more than 35% of all cancers, it is highly significant for PDAC (>95% of tumors with Kras mutations; ref. 22). Kras-driven cellular and animal tumor models have a positive impact on our understanding of the origins and
progression of various stages of PDAC. Despite this knowledge, Kras has remained an elusive target for more than 30 years with very little progress in the development of Kras-targeted agents for use in cancer treatment, especially PDAC (23). The absence of any druggable cavity in the Kras protein structure has compounded the problem. Additional strategies to target downstream effectors of Kras have been touted as potential avenues of anticancer intervention. Inhibition of Kras membrane association (24), farnesyltransferases (25), Raf/MEK/ERK (26), and PI3K/AKT/mTOR (27) pathways and RalGEF/Ral (28) have all been evaluated as strategies to tame this elusive oncogenic target. However, none have lived up to their potential and in most instances have failed to translate into successful clinical therapies. These failures highlight the urgent need to identify novel therapeutic strategies that can expose the impregnable oncogenic Kras fortress.

The PAKs are uniquely positioned at the junction of several oncogenic signaling pathways (29). There are 2 groups of PAKs (Group 1: PAK1–3 and Group 2: PAK4–6). Their importance can be illustrated by the fact that aberrant expression or mutational activation of different PAK isoforms frequently occurs in human cancers (30). A number of studies have clearly demonstrated that increased PAK activity drives multiple cellular processes feeding cancer development, sustenance, and progression. The PAK family members are key effectors of the Rho family of GTPases downstream of Ras, which act as regulatory switches controlling critical cellular processes such as cell motility, proliferation, and survival (6). Notably, PAK4 is a key downstream effector of Cdc42 (cell division control protein 42 homolog; ref. 7). Gene amplification at chromosome 19q13 (i.e., PAK4) has been recorded in PDAC and has been linked to poor overall survival. PAK genes can be hyperactivated by mutations in upstream regulators such as Rac or its exchange factors. Copy number alteration analyses have shown amplification of PAK4 in cancer patients samples (8). As presented

![Figure 6.](image-url)

**Figure 6.** PAM shows remarkable antitumor activity in animal tumor xenograft of PDAC. A, L3.6pl cells were grown as subcutaneous tumors in ICR-SCID mice (Taconic). For the experimental groups, 10 mice were treated with about 30-mg tumors at subcutaneous site bilaterally. After 1 week, the mice were randomly divided into 2 groups: control (vehicle) and treated (KPT-9274) relative to PAM analog with superior pharmacokinetic properties given at 140 mg/kg intravenously once a day 5 days a week for 4 weeks. At day 21 of the experiment, KPT-9274 was administered to the control arm at a dose of 140 mg/kg intravenously once a day 5 days a week for an additional week. Note: suppression of tumors in the control arm. The tumors were excised from one mouse for immunohistochemical analysis, and H&E staining confirmed the presence of tumors. B, Mice were monitored for body weight loss and did not show any outward signs of toxicity. C, Photographs showing control versus KPT-9274-treated mice.
PAK4 Inhibitors for Pancreatic Cancer Therapy

here, our analyses of the PAK4 expression patterns in regular and stem cell marker–enriched flow-sorted PDAC cells point to a role in supporting the stemness of CSCs.

In this study, we used a candidate group of proteins downstream of PAK4 to demonstrate antitumor activity of KPT-9274 and other PAMs. In agreement with our hypothesis, we observe selective inhibition of p-PAK4 with no effect on group 1 PAKs. In addition, downregulation of cyclin D1 supports the cell-cycle arrest of PAMs. Further work to characterize the endogenous proteins (targeted by PAMs) regulating PAK4 is ongoing. In this direction, a recent study demonstrates that Inka1 acts as an endogenous inhibitor of PAK4 (31). We are currently evaluating the impact of PAMs on Inka1 expression and its consequence on PAK4 protein expression. Additional downstream targets influenced by PAM activity were recently described by our collaborators in kidney and esophageal tumor models (14, 17). These findings are highly significant and further support the rational development of KPT-9274 for treatment of chemotherapy-resistant tumors.

Most of the pharmaceutical companies developing PAK4 inhibitors have focused on Type I or ATP-competitive inhibitors. Earlier attempts to target PAK4 resulted in the development of PF-03758309 which was prematurely discontinued in a single clinical trial due to lack of efficacy as well as undesirable pharmacokinetic characteristics (<1% human bioavailability; NCT00932126; ref. 32). A major drawback of PF-03758309 was its propensity as a substrate of the multidrug transporters contributing to its low bioavailability in humans (33). Another group developed a potent PAK4 inhibitor, LCH-7749944, which was not only effective in suppressing PAK4 activity but also could interfere with plasticity and filapodia formation in gastric cell lines (34). However, its clinical utility has yet to be explored. Nonsynthetic plant–derived natural agents have also been explored for their ability to inhibit PAK4 activity. Glauarubinone isolated from the seeds of the Simarouba glauca tree was also shown to inhibit PAK4 protein expression in Kras-driven pancreatic cancer cellular and animal models (35). Glauarubinone could also synergize with standard chemotherapeutic gemcitabine in a PAK4-dependent manner. However, it should be noted that PAK4 is not the primary target of glauarubinone which was originally developed as an antimalarial agent. Being a natural agent, it most likely has pleiotropic effects complicating its development as a therapeutic agent. A recent group, using high-throughput screening and structure-based drug design, identified a novel PAK4 inhibitor, KY-04031 (36). Unfortunately, KY-04031 showed very low binding affinity toward PAK4 and required high micromolar concentrations to inhibit cell proliferation in PAK4-dependent manner. This agent serves only as a scaffold for future development of novel PAK4 agents. Aside from these preclinical agents, the field has not produced any PAK4-targeted clinical stage compounds. PAK proteins possess a very flexible ATP-binding cleft in their structure rendering the design of a specific ATP-competitive inhibitor very difficult. These could be some of the reasons for the lack of any objective responses with Type I kinase inhibitors in the clinic and therefore drive the urgency for the discoveries of alternative drugs.

In the absence of any clinically successful ATP-competitive Type I PAK4 inhibitors, the development of allosteric or Type II modulators against PAK4 becomes a distinct and attractive approach to control this important player in the Kras pathway. However, non–ATP-competitive inhibitor development is an underappreciated area of research. As presented in this article, the PAMs could fill this void and become a much needed alternative in this stagnated field.

KPT-9274 is not a substrate of the multidrug transporters which plagued the pharmacokinetic properties of PF-03758309 (data not shown). This is certainly an advantage over its predecessor that has been halted from further clinical evaluation. Increased clinical utility of PAMs may be realized in the synergism

Figure 7.
PAM suppresses PDAC CSC tumors. CD44+/CD133+/EpCAM+ (triple marker–positive cells) were isolated as the CSLCs from human pancreatic cancer cell line MiaPaCa-2 by the FACS technique and cultured in the serum-free sphere formation medium (1:1 DMEM/F-12 K medium plus B-27 and N-2 supplements, Invitrogen) to maintain its undifferentiated status. The animal protocol was approved by the Animal Investigation Committee, Wayne State University (Detroit, MI). Female ICR-SCID mice (4 weeks old) were purchased from Taconic Farms and fed Lab Diet 5021 (Purina Mills, Inc.). Initially, 1 × 105 CD44+/CD133+/EpCAM+ (triple marker–positive cells) MiaPaCa-2 cells were implanted in xenograft mouse at the subcutaneous site bilaterally. One mouse was sacrificed after 2 to 3 weeks to confirm the growth of the tumor. A, Mice harboring CSC tumors bilaterally were administered vehicle, gemcitabine, or nab-paclitaxel (at indicated doses intravenously once a week for 2 weeks). B, Mice harboring MiaPaCa-2 CSC were then randomly divided into 2 groups with 6 animals in each group: (i) vehicle control and (ii) KPT-9274 (140 mg/kg once daily intravenously). After the termination of the treatment arm, the control group was exposed to KPT-9274 140 mg/kg intravenously at day 32 and these mice were followed till day 49. Note almost complete reduction in KPT-9274 arm and statistically significant reduction in the control arm that was exposed to KPT-9274 at late time points.
with low-dose gemcitabine or oxaliplatin leading to improved antitumor activity with no observed toxicity in mice. Aside from pancreatic cancer models, PAMs have also shown activity in other hematologic malignancies in vitro (see Supplementary Fig. S1 for the antitumor activity of PAM analog across >100 different cancer cell lines).

Most interestingly, KPT-9274 and analogs are effective against pancreatic cancer stem cells that undergo EMT. These are highly significant findings, as Cdc42 and Rho GTPases are known to promote motility pathways such as directional filapodia migration (37, 38). It should be noted that cellular motility (such that observed during EMT) is catalyzed by a multitude of mechanisms that includes activation through the NAD⁺ biosynthetic enzyme, nicotinamide phosphoribosyltransferase (NAMPT); genome; ref. 39). NAMPT mediates directionally persistent migration of vascular smooth muscle cells (SMC) and cancer cells (40). Closing the loop, NAMPT logic shown to activate Cdc42 (40). Being downstream of Cdc42, PAMS can be in principle dual inhibitors for both NAMPT and PAK4. The PAK4/NAMPT dual inhibitory activity of PAMs was recently described (14). Given these recent publications, we are currently evaluating the anti-NAMPT activity of PAMS in PDAC. Nevertheless, these are beyond the scope of this article.

Being the direct effectors of oncogenic Kras, PAK genes are high value targets. As shown in this study, the PAK4 protein acts as a central signaling node in cancer cells, and when inhibited, it can have a meaningful impact on Kras pathway in preclinical models of PDAC. Despite several serious attempts to develop PAK4-targeted drugs, a clinically viable agent has not come to fruition. This is exemplified by the failure of the first-in-class Type I ATP-competitive PAK inhibitor (PF-03758309) and highlights the unmet clinical challenge of therapeutically targeting PAK4 and other related kinases. KPT-9274 described in this study has recently entered phase I clinical evaluation in patients with advanced solid malignancies and non–Hodgkin lymphoma (NHL; clinicaltrials.gov; NCT02702492), can be evaluated in patients with PDAC directly, and warrants further evaluation in other cancers.

Disclosure of Potential Conflicts of Interest

W. Senapedia and E. Baloglu hold patent and equity stocks in Karyopharm Therapeutics Inc. Y. Landesman is the Senior Director and Head of Scientific Affairs at Karyopharm Therapeutics Inc. M. Kauffman is the CEO of and has ownership interest (including patents) in Karyopharm Therapeutics Inc. S. Shacham is CSO of and has ownership interest (including patents) in Karyopharm Therapeutics Inc. No potential conflicts of interest were disclosed by the other authors.

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# Molecular Cancer Therapeutics

## Novel p21-Activated Kinase 4 (PAK4) Allosteric Modulators Overcome Drug Resistance and Stemness in Pancreatic Ductal Adenocarcinoma


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