Targeting Nuclear Pore Complex for Pancreatic Cancer Therapy

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ABSTRACT

The incidence of pancreatic neuroendocrine tumors (PNETs) has been increasing considerably likely due to improved awareness, classification, and diagnostic modalities. Frequent mutations in MEN1 (44%), DAXX/ATRX (43%), mTOR (15%) pathway genes and Von Hippel Lindau disease (VHL) alongside several other hereditary disorders are observed in PNETs. This disease remains an unmet clinical problem as the available targeted therapies are limited to mTOR inhibitor everolimus and multitargeted RTK inhibitor sunitinib that lack objective response. Evidence from the literature suggests that p21 activated kinase 4 (PAK4) protein regulates a myriad of signaling pathways associated with proliferation and therapy resistance in PNETs including mTORC1, mTORC2, PI3K, IGF-1; thus making it a viable therapeutic target for everolimus resistant PNETs. Similarly, intracellular Nicotinamide Phosphoribosyltransferase (NAMPT), which is the rate-limiting enzyme in the NAD biosynthesis pathway is recognized to regulate mTOR signaling pathway through activation of the energy sensor AMPK. In this study, we found that compared to normal HPNE cells, patient-derived tumor tissue and PNET cell lines exhibit hyperactivation of PAK4 and NAMPT. PNET cell lines QGP-1 and BON-1 were subjected to siPAK4 and siNAMPT RNA interference (RNAi), or PAK4-NAMPT dual inhibitor KPT-9274 (currently in Phase I clinical study NCT02702492) in the presence or absence of mTOR inhibitor everolimus. SiPAK4-SiNAMPT RNAi suppressed proliferation in PNET cell lines. KPT-9274 was effective in reducing proliferation and restored mTOR inhibitor (everolimus) sensitivity. Isobologram analysis showed that KPT-9274 could synergistically enhance the anti-tumor activity of everolimus in PNET cell lines (combination index <1). Molecular analysis of combination treatment showed down-regulation of known everolimus resistance drivers such as mTORC1, mTORC2, PI3K, ERK, FAK, RICTOR, β-catenin. KPT-9274 suppressed NAD pool and ATP levels in PNET cell lines. Importantly, KPT-9274 given orally at 150 mg/kg 5 days a week for 4 weeks dramatically inhibited the growth of BON-1 sub-cutaneous xenograft tumors. Combination KPT-9274 (150 mg/Kg) and everolimus (2.5 mg/Kg) 3 days/week for 4 weeks significantly reduce QGP-1 sub-cutaneous tumor growth. This is the first report demonstrating the role of PAK4 and NAMPT in PNET therapy resistance. Our investigations demonstrate that PAK4 and NAMPT are two viable therapeutic targets in the difficult to treat PNETs that warrant further clinical investigations.

OBJECTIVE

The goal of this project is to demonstrate the importance of PAK4 and NAMPT in PNETs subsistence. We also evaluate the anti-cancer activity of the oral dual inhibitor of PAK4 and NAMPT (KPT-9274) in PNET cell lines. We further investigate whether KPT-9274 in combination with Everolimus is a feasible strategy for the treatment of intractable PNET tumors. PNET cell line BON-1 was obtained under an MTA from Dr. Townsend at University of Texas Medical Branch, Galveston, TX, USA. QGP-1 cell line was purchased from the Japanese Collection of Research Bioresources Cell Bank (JRCB, Osaka, Japan). Under an IRB approved protocol, we obtained a PNET tumor tissue with matched control from the same patient from Karmanos Cancer Institute. PNET cell lines were exposed to KPT-9274 single agent and in combination with Everolimus. Cell growth inhibition was evaluated using MTT, clonogenic and cell Titer-Glo assays. Apoptosis was analyzed using Annexin V FITC and 7-Aminoactinomycin D (7AAD) assay. Protein expression and mRNA expression changes were evaluated using Western blot and RT-PCR. The antitumor activity of KPT-9274 was also evaluated in PNETs subcutaneous xenograft. Metabolomics alteration post KPT-9274 treatment was examine using liquid Chromatography-mass spectrometry.

PAK4 AND NAMPT EXPRESSION IN PNETs

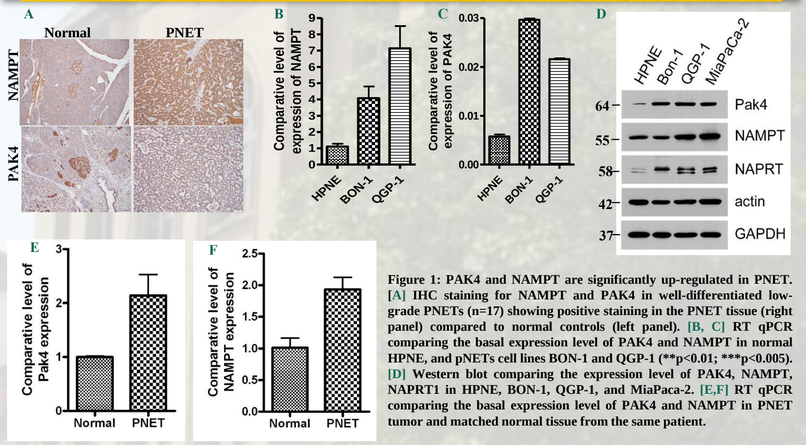

Figure 1: PAK4 and NAMPT are significantly up-regulated in PNET. [A] IHC staining for NAMPT and PAK4 in well-differentiated low-grade PNETs (n=17) showing positive staining in the PNET tissue (right panel) compared to normal controls (left panel). [B, C] RT qPCR comparing the basal expression level of PAK4 and NAMPT in normal HPNE, and pNETs cell lines BON-1 and QGP-1 (p<0.01; ***p<0.005). [D] Western blot comparing the expression level of PAK4, NAMPT, NAPRT1 in HPNE, BON-1, QGP-1, and MiaPaca-2. [E,F] RT qPCR comparing the basal expression level of PAK4 and NAMPT in PNET tumor and matched normal tissue from the same patient.**

PAK4 AND NAMPT RNA INTERFERENCE IN PNETS CELL LINES

Figure 2: PAK4-NAMPT RNA interference inhibits the growth of PNET cellular models and suppresses PNET signaling. [A] Western blot showing PAK4 and NAMPT knockdown post RNA interference in QGP-1. [B] Colony formation assay to show that PAK4-NAMPT RNA interference induces long term growth inhibition in pNET cell lines [C,D] Animal study showing significant growth inhibition of siPAK4-siNAMPT silencing in BON-1 tumor.

PAK4 AND NAMPT DUAL INHIBITOR ACTIVITY IN PNETs CELL LINES IN VITRO

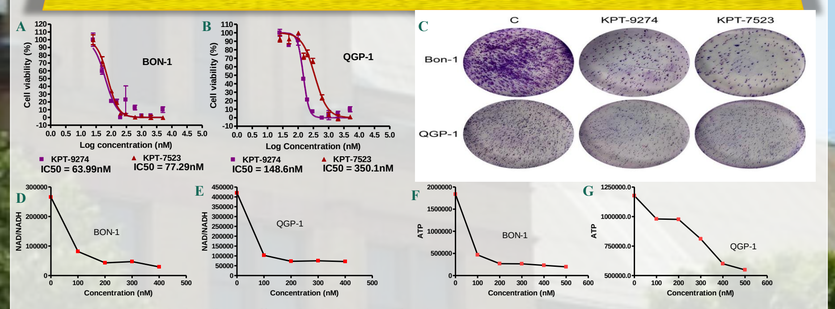

Figure 3: PAK4-NAMPT dual inhibitors effectively block cell proliferation and cause energy collapse in PNET cellular model. [A-B] Graphs representing growth curves in the presence of KPT-9274 and analog KPT-7523 in BON-1, QGP-1 (72hrs MTT assay). [C] Colony formation assay post KPT-9274 and analog KPT-7523 in BON-1 and QGP-1. [D] NAD cell titer-Glo assay showing a reduction of NAD pool after KPT-9274 treatment in BON-1 and GP-1. [E] ATP cell Titer-Glo assay showing a reduction of ATP pool level after PAK4 and NAMPT inhibition.

KPT-9274-EVEROLIMUS SYNERGY ANALYSIS IN PNET CELLS LINES

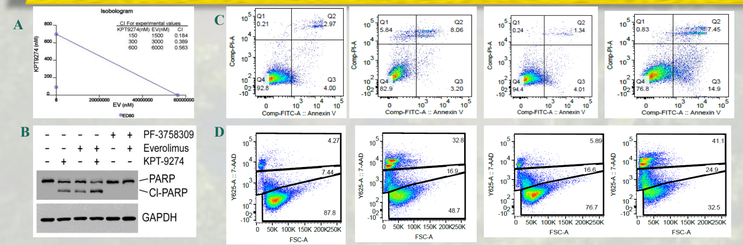

Figure 4: KPT-9274 synergizes with the FDA approved mTOR inhibitor everolimus. [A] Isobologram showing the synergistic interaction of KPT-9274 with everolimus (EV) at 72hrs using MTT assay in QGP-1 with CI<1 for all doses tested. [B] Western blot showing robust apoptosis in KPT-9274 + Everolimus combination. [C] Annexin V FITC apoptosis analysis in flow cytometry for 72 hrs using 600 nM of KPT-9274; 6μM everolimus and their combination. [D] 7AAD staining apoptosis at 72 hrs treatment using same drug concentration as in C.

MOLECULAR ANALYSIS POST KPT-9274-EVEROLIMUS TREATMENT

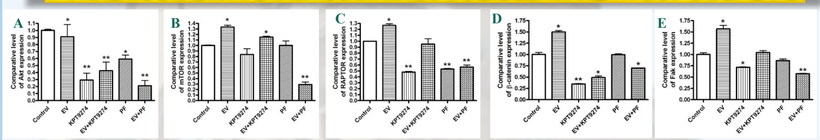

Figure 5. Molecular analysis of the KPT-9274-everolimus combination. [A–E] RT qPCR in QGP-1 cells showing the down-regulation of survival factors after KPT-9274 treatment. Cells were seeded at a density of 50,000 cells per well in six-well plates in duplicate and exposed to KPT-9274 (600 nM); PF-3578309 (300 nM); everolimus (6 μM) or their combination for 72 h. After each treatment period, pooled RNA from each group was isolated and subjected to real-time qPCR. The expression level of Akt, mTOR, RAPTOR, β-catenin, and focal adhesion kinase (FAK) mRNA was normalized with GAPDH mRNA. * $p < 0.05$; ** $p < 0.01$.

METABOLOMIC ANALYSIS POST KPT-9274 TREATMENT

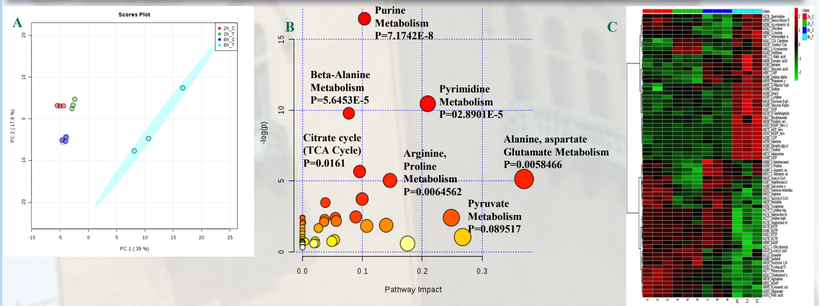

Figure 6: Metabolomics analysis of KPT-9274 in PNET cell lines. [A] Principal component analysis. [B] Pathway analysis showing changes of key component of the energy system in QGP-1. [C] Heat map (respectively) showing alteration of the metabolism post KPT-9274 treatment in QGP-1. Alteration occurs 8 hours post treat at 600 nM of KPT-9274.

KPT-9274 AND EVEROLIMUS PRE-CLINICAL EFFICACY TRIAL IN PNETs

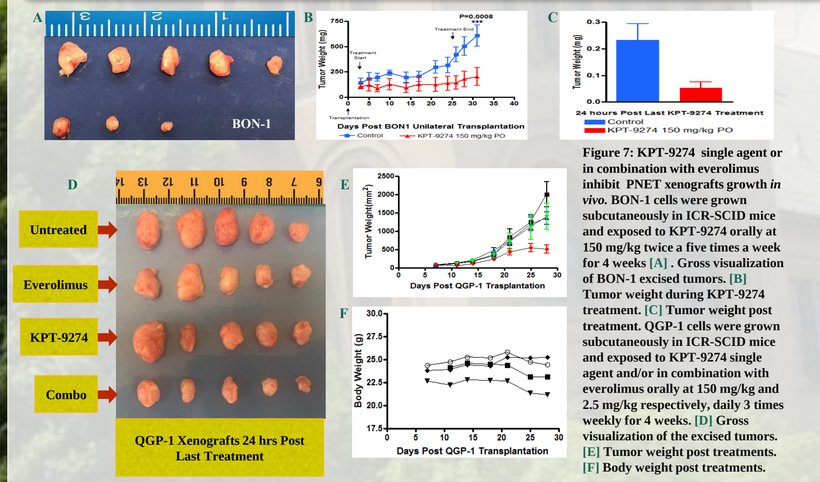

Figure 7: KPT-9274 single agent or in combination with everolimus inhibit PNET xenografts growth *in vivo*. BON-1 cells were grown subcutaneously in ICR-SCID mice and exposed to KPT-9274 orally at 150 mg/kg twice a five times a week for 4 weeks [A] . Gross visualization of BON-1 excised tumors. [B] Tumor weight during KPT-9274 treatment. [C] Tumor weight post treatment. QGP-1 cells were grown subcutaneously in ICR-SCID mice and exposed to KPT-9274 single agent and/or in combination with everolimus orally at 150 mg/kg and 2.5 mg/kg respectively, daily 3 times weekly for 4 weeks. [D] Gross visualization of the excised tumors. [E] Tumor weight post treatments. [F] Body weight post treatments.