PAK4 Allosteric Modulators (PAMs) Repress the Wnt/β-catenin Signaling Pathway and Tumor Growth

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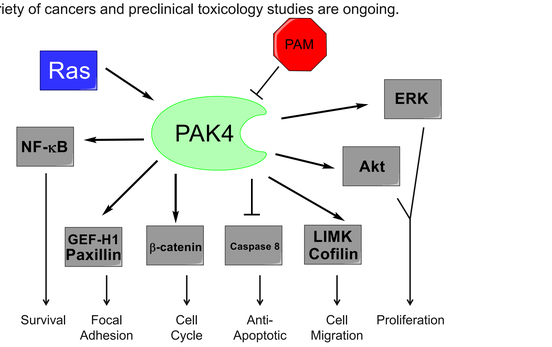
Abstract

Background: Wnt/β-catenin signaling is a ubiquitous pathway conserved throughout evolution and plays a role in embryonic and cancer development. However, this pathway has proven to be difficult to target therapeutically. Another intractable target is oncogenic Ras which is highly mutated in many cancer types, including pancreatic, colon and lung. An available target at the intersection of both of these genes is p21-Activated Kinase 4 (PAK4). PAK4 (1 of 6 PAK proteins) that is downstream of the Ras oncogene and a direct kinase that stabilizes transcriptional activity of β-catenin. Therefore therapeutically targeting PAK4 could be beneficial in a broad range of cancer types. PAK4 Allosteric Modulators (PAMs) represent a novel and selective class of compounds that inhibit PAK4 allosterically.

Methods: Flow cytometry and CellTiter AQueous One assay (MTS) were used to determine compound effects on cell cycle distribution and proliferation. CCLE, COSMIC and other databases were used for bioinformatics analysis of mutations in cancer genes. Deep sequencing and qPCR were used to analyze mRNA expression profiles. Proteomic platforms – KinomeScan, PTScan (Cell Signaling) and immunoblots were used to study phosphorylation signatures of whole cell proteins and total protein steady state levels.

Results: We identified selective, orally bioavailable small molecules, (PAMs; KPT-6604, KPT-7523, KPT-8752 and KPT-9274) which demonstrated anti-tumor activity in a variety of cancer cell lines (IC_{50} values from 0.005 – 1 μM). Bioinformatics revealed that sensitivity to PAMs was directly correlated with mutations in APC or N-ras and inversely correlated with mutations in β-catenin, K-ras, or PI3K. KPT-9274 and other PAMs reduced phosphorylation and steady state levels of PAK4 protein while reducing Phospho-S675 and total β-catenin, Wnt5, Phospho- and total LRP6, Dvl2, and Axin1. PAMs also reduced β-catenin transcriptional activity (i.e. CCND1, WNT5A, and WNT10A). PAMs arrested cancer cell cycle at the G1 and G2 phases and induced apoptosis through Caspase and PARP cleavage. KPT-9274 (~100 mg/kg BIDx5 orally) has demonstrated potent anti-tumor activity against hematological (Z-138, Molt-4, MM1S) and solid (MDA-MB-231, MDA-MB-468, H520, Hep 3B and Colo-205) xenograft models in mice. PAM-treated xenografts showed reduction of PAK4, β-catenin and cyclin D1 proteins.

Conclusions: PAK4 represents a novel anti-cancer target at the crossroads of Ras and Wnt/β-catenin signaling. We have identified selective small molecule PAMs with anti-tumor activity both in vitro and in vivo. These allosteric modulators induce tumor cell growth arrest and apoptosis. Bioinformatics helped identify potential predictive markers in the Wnt signaling pathway while deep sequencing and proteomics revealed possible PDn markers. Based on the in vitro and in vivo activity, KPT-9274 may be beneficial for the treatment of a wide variety of cancers and preclinical toxicology studies are ongoing.



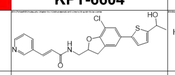
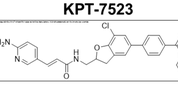
PAMs	KPT-6604	KPT-7523	KPT-8752	KPT-9274
Cell Line MTT (IC_{50} μM)				
Z-138	0.04	0.03	0.02	0.008
PC3	0.22		0.14	0.06
Colo-205	0.24		0.16	0.04
NIH 3T3	0.3	1.4	>10	2.5
HCT116	3.22		>10	>10

Figure 1: PAK4 Allosteric Modulators inhibit cancer cell growth. A novel class of PAK4 inhibitors display anti-tumor activity in vitro while sparing normal (NIH 3T3). Cells were assessed by MTS assay 72 hours after treatment with various structural analogs of the early lead compound, KPT-6604. The clinical candidate compound is KPT-9274.

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Bioinformatics Analysis Suggest PAM In Vitro Sensitivity is Predicted to be though Ras and Wnt Pathways

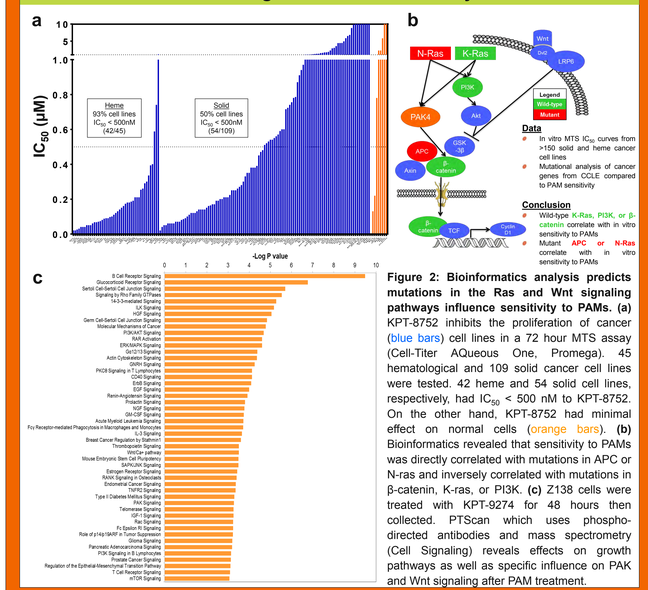

Figure 2: Bioinformatics analysis predicts mutations in the Ras and Wnt signaling pathways influence sensitivity to PAMs. (a) KPT-8752 inhibits the proliferation of cancer (blue bars) cell lines in a 72 hour MTS assay (Cell-Titer AQueous One, Promega). 45 hematological and 109 solid cancer cell lines were tested. 42 heme and 54 solid cell lines, respectively, had IC_{50} < 500 nM to KPT-8752. On the other hand, KPT-8752 had minimal effect on normal cells (orange bars). **(b)** Bioinformatics revealed that sensitivity to PAMs was directly correlated with mutations in APC or N-ras and inversely correlated with mutations in β-catenin, K-ras, or PI3K. **(c)** Z138 cells were treated with KPT-9274 for 48 hours then collected. PTScan which uses phospho-directed antibodies and mass spectrometry (Cell Signaling) reveals effects on growth pathways as well as specific influence on PAK and Wnt signaling after PAM treatment.

PAMs Display In Vivo Efficacy in Colo-205 with a Reduction in Biomarkers

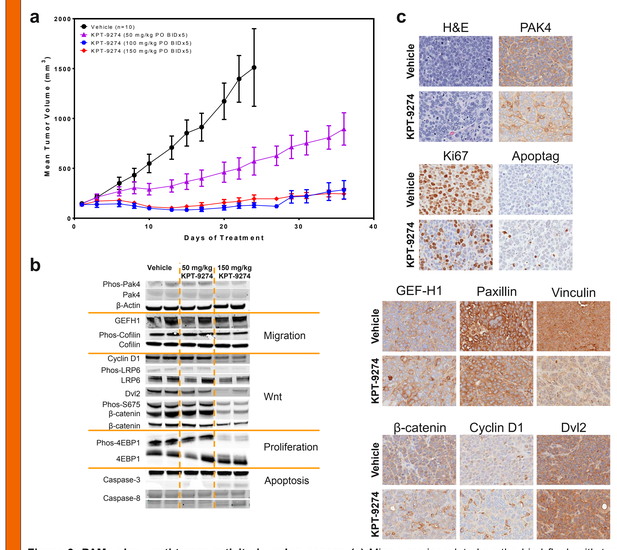

Figure 6: PAMs show anti-tumor activity in colon cancer. (a) Mice were inoculated on the hind flank with tumor cells. Mice began treatment when average Colo-205 tumor volume reach ~100 mm^3 per group (n = 10 animals/dose group). Mice were treated either with vehicle or oral PAM (KPT-9274) twice daily (5 days/week) without major toxicity (minimal or no side effects or weight loss). **(b)** Tumors were collected 2 hours post-dose after 3 weeks of dosing with oral KPT-9274. Frozen tumors were grounded up and lysed in a Tissue Lyser (Qiagen) using the T-PER buffer (Pierce). **(c)** Colo-205 tumors were collected and formalin fixed 2 hours post-dose. Cellular biomarkers of PAK4 and Wnt/β-catenin signaling were dramatically changed after treatment with oral KPT-9274. There was a reduction in proliferation (Ki67) and an increase in apoptosis (Apoptag).

Clinical Candidate, KPT-9274, Potently Cytotoxic to Cancer Cells and Inhibits PAK4 and Wnt/β-catenin Signaling In Vitro

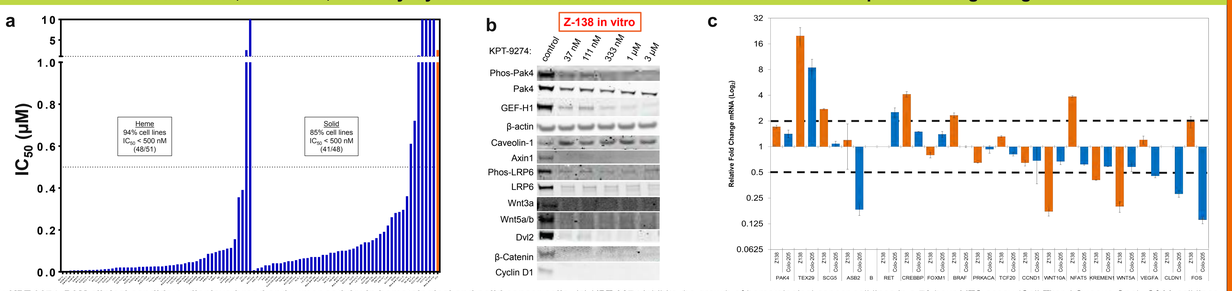

Figure 3: KPT-9274, PAM clinical candidate, displays broad anti-tumor activity in hematological and solid cancer cells. (a) KPT-9274 inhibits the growth of hematological cancer cell lines in a 72 hour MTS assay (Cell-Titer AQueous One). Of 99 cell lines tested, 89 cell lines had IC_{50} values < 500 nM (90%). **(b)** Z-138 (MCL) cancer cells were treated with various amounts of KPT-9274 (IC50 = ~10 nM) for 72 hours then lysed for western blot analysis. Treatment of cells with KPT-9274 showed decreases in PAK4 protein and signaling along with key downstream effectors of cell cycle (cyclin D1), cell migration (GEF-H1), and Wnt signaling (β-catenin, Dvl2, and LRP6). **(c)** Z-138 and Colo-205 cells were treated with 1 μM KPT-9274 for 48 hours then total RNA was collected. RT-PCR was analyzed by cDNA creation then measured by Taqman probes using Via VII from Invitrogen. KPT-9274 treatment increases the PDn marker TEX29 transcripts in both cells lines and decreases ASB2 in Colo-205. There is also a marked repression of β-catenin responsive genes such as WNT5A, WNT10A, KREMEN1, and cyclin D1. The overall effects on mRNA expression are more significant in the Colo-205 then at Z138 at 48 hours.

Orally Bioavailable PAMs Display Broad In Vivo Efficacy

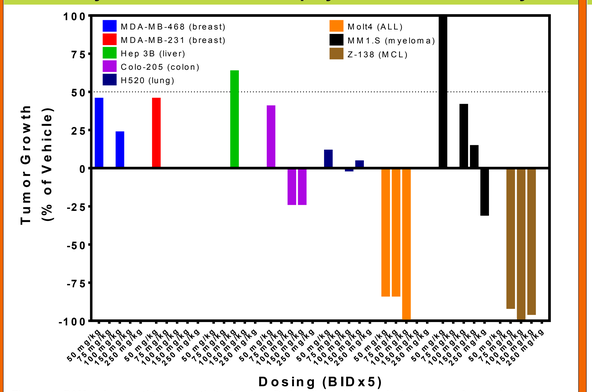

Figure 4: PAMs show anti-tumor activity in vivo. Both solid and hematological xenografts in mice show dose response to KPT-9274 oral treatment. Mice were inoculated with different cancer cell lines in the hind flank. When the tumor reached 100 – 200 mm^3 vehicle or oral KPT-9274 treatment was begun. Mice were treated with various amount of PAM at BIDx5 per week for ~4 weeks. The best response was used to calculated growth repression or regression of tumors when compared to vehicle tumors. The hematological tumors showed high levels of regression with several or all animals "cured" of tumors. The solid tumors scored high for growth repression.

PAMs Display In Vivo Efficacy in PC3 Xenograft in Rats

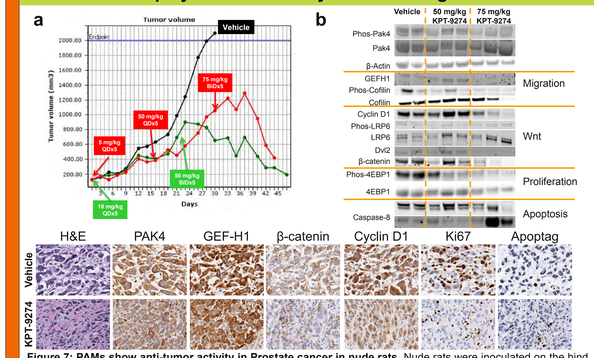

Figure 7: PAMs show anti-tumor activity in Prostate cancer in nude rats. Nude rats were inoculated on the hind flank with PC3 tumors. **(a)** Rats began treatment when average tumor volume reach ~100 mm^3 (n = 8/group). Rats were treated either with vehicle or oral PAM (KPT-9274) at 5 and 10 mg/kg daily (5 days/week) with dose escalations as marked. Tumor regression occurred after escalating to 50 and 75 mg/kg BIDx5 with stable disease achieved with 10 and 50 mg/kg QDx5. **(b)** Tumors were collected 2 hours post-dose at the end of the study. Frozen tumors were grounded up and lysed. **(c)** IHC staining of formalin fixed tumors from **(b)**.

PAMs Display In Vivo Efficacy in Z-138 with a Reduction in Biomarkers

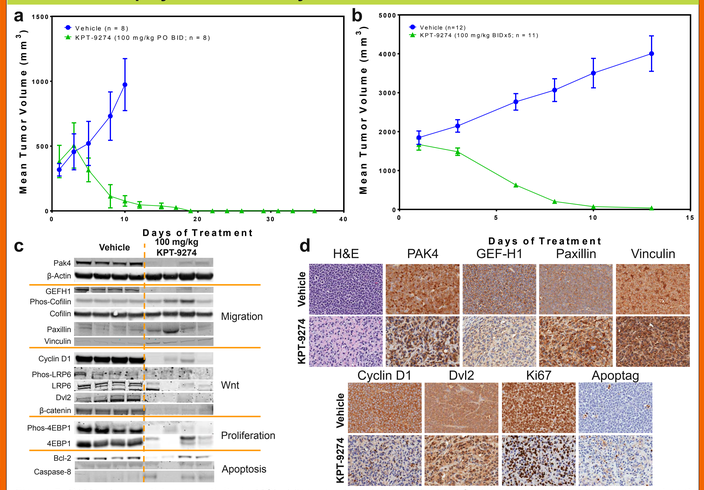

Figure 5: PAMs show anti-tumor activity in MCL. Mice were inoculated on the hind flank with tumor cells. Mice began treatment when average Z-138 tumor volume reach **(a)** ~300 or **(b)** 1500 mm^3 per group (n = 8-12 animals/dose group). Mice were treated either with vehicle or oral PAM (KPT-9274) twice daily (5 days/week). **(a)** Tumor regression("cures") occurred after ~2 weeks of dosing. After stopping treatment on Day 21, 8 out of 8 animals remained tumor free for more than 2 weeks before euthanasia. **(b)** Large tumors regressed to near full elimination after ~2 weeks of dosing. **(c)** Tumors were collected 2 hours post-dose of 100 mg/kg oral KPT-9274 administered for ~1 week. Frozen tumors were grounded up and lysed in a Tissue Lyser (Qiagen) using the T-PER buffer (Pierce). **(d)** Formalin fixed tumors from **(c)** were processed and stained for immuno-histochemistry. Cellular biomarkers of PAK4 and Wnt/β-catenin signaling were dramatically changed after treatment with oral KPT-9274. There was a reduction in proliferation (Ki67) and an increase in apoptosis (Apoptag).

Conclusions

- PAMs demonstrate anti-tumor activity across a broad range of hematological and solid malignancies in vitro while sparing normal cells.
- PAMs decrease PAK4 protein levels and signaling pathways of treated cells.
- PAMs decrease activity of Wnt/β-catenin both in vitro and in vivo.
- PAMs are orally bioavailable and display anti-tumor activity in hematological and solid xenograft mouse and rat models with excellent tolerability.
- **KPT-9274** is our clinical candidate with IND expected in 2H2015