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Inhibiting p21-Activated Kinase Induces Cell Death in Vestibular Schwannoma and Meningioma via Mitotic Catastrophe

Melania E. Mercado-Pimentel, Ph.D.^{1,2,3}, Craig Miller, M.D.^{1,2}, Daniela N. Rolph, B.S.^{1,2}, Edrick F. Villalobos, B.S.^{1,2}, Allison M. Dunn, B.S.^{1,2}, Prithvi M. Mohan^{1,2}, Suzu Igarashi, B.S.^{1,2}, Xiangdang Liu, M.D.^{1,2}, Macken Yrun-Duffy^{1,2}, Neal K. Patel^{1,2}, Cecilia M. Read, B.S.^{1,2}, Ross H. Francis, B.S.^{1,2}, Adelina Isabella Lane^{1,2}, Swaroop Muruges^{1,2}, and Abraham Jacob, M.D.^{1,2,3,4}

¹Ear Institute University of Arizona

²Department of Otolaryngology-Head & Neck Surgery College of Medicine University of Arizona

³Arizona Cancer Center

⁴BIO5 Institute University of Arizona

Abstract

Hypothesis—p21-Activated Kinase (PAK) regulates signaling pathways that promote cell survival and proliferation; therefore, pharmacological inhibition of PAK will induce cell death in vestibular schwannomas (VS) and meningiomas.

Background—All VS and many meningiomas result from loss of the neurofibromatosis type 2 (NF2) gene product merlin, with ensuing PAK hyperactivation and increased cell proliferation/survival.

Methods—The novel small molecule PAK inhibitors PI-8 and PI-15 – tested in schwannoma and meningioma cells – perturb molecular signaling and induce cell death. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), flow cytometry and TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay analyzed PAK inhibitors' effect on cell viability, cell cycle and cell death, respectively. Western blots evaluated activation and expression of cell proliferation, apoptotic, and mitotic catastrophe markers. Light microscopy evaluated cell morphology, and immunocytochemistry analyzed cellular localization of phospho-Merlin and ATG5 (autophagy-related protein).

Results—Treatment with PI-8 and PI-15 decreased cell viability at 0.65–3.7 μ M IC₅₀ in schwannoma and meningioma cells. TUNEL and immunocytochemistry studies show that PI-8 and PI-15 induce mitotic catastrophe but not apoptosis in HEI193 cells while in BenMen1 cells, PI-8 induces autophagy and mitotic catastrophe. PI-15 induces apoptosis in BenMen1 cells. PAK inhibitor treated cells show phospho-Merlin localized to over-duplicated centrosomes of dividing cells, multiple enlarged nuclei, and misaligned/missegregated chromosomes – markers for mitotic catastrophe. Increased ATG5 levels in the nucleus confirmed this cell death type. PI-8 and PI-15

inhibits PAK in both cell lines. However, only PI-15 inhibits AKT (v-Akt Murine Thymoma Viral Oncogene Homolog) in BenMen1 cells.

Conclusion—PAK inhibitors induce cell death in schwannoma and meningioma cells, at least in part, by mitotic catastrophe.

Introduction

Neurofibromatosis type 2 (NF2) is an autosomal-dominant familial syndrome caused by loss-of-function mutation in the NF2 gene, which encodes the tumor suppressor protein, Merlin(1). NF2 disease is characterized by the development of bilateral intracranial benign tumors known as vestibular schwannomas (VS) and meningiomas, among other neoplasias (2). Meningiomas are tumors derived from the meninges, which 30% to 60% of the cases are benign tumors associated with the NF2 gene inactivation or mutations (3). Vestibular Schwannomas (vestibular neurilemmomas, acoustic neurinomas, acoustic neuroma) originate from Schwann cells surrounding the vestibular branch of the VIII nerve. They can also appear as unilateral tumors, which encompasses 90 % of all VS and are associated with somatic NF2 gene mutations(1, 2). VS can cause hearing loss, tinnitus and imbalance among other symptoms. Current treatment options include observation, surgery or radiation. However, the last two present serious risks, including, cerebrospinal fluid leaks, meningitis, intracranial hemorrhage, stroke, comma, latent tumor growth, and secondary skull malignances, among others (4). The lack of FDA approved chemotherapeutic agents is linked to the poor understanding of the molecular mechanisms of NF2-associated tumor growth.

However, several studies have demonstrated that p-21 activated kinase (PAK) has a role in cell survival and apoptosis signaling pathways as well as in cancer initiation and progression (5–7). These serine/threonine protein kinases, stimulated by Rac and Cdc42, are involved in organizing actin and intermediate filaments, enhancing cell proliferation, and inhibiting apoptosis (8–12). Recent studies suggest that PAK and Merlin reciprocally regulate each other's function influenced by cellular adhesion and cell density (7, 13). Merlin binding to PAK inhibits RAC/Cdc42-PAK interaction, thus, inactivating PAK, while active PAK phosphorylates Merlin at ser518, and therefore causing cell transformation (14–16). PAK is known to aid in the recruitment of AKT to the membrane, and to phosphorylate PDK1, which activates by phosphorylation the AKT signaling pathway in cell proliferation during tumor growth (17). Therefore, PAK has been suggested a target for drug development to treat NF2-associated tumors (18–20). Because Merlin loss results in aberrant PAK activation, targeting PAK by using novel small molecule inhibitors may represent a viable treatment strategy for vestibular schwannomas and meningiomas.

The two novel PAK inhibitors, PI-8 and PI-15, were derived from AR12 (OSU-03012), which is a PDK1 inhibitor, and at a lower concentration it acts as PAK inhibitor in different types of cancer cells and in VS cells (21–25). Binding of AR12 is located in the ATP binding pocket of PAK and by using computer modeling AR12 was structurally altered to reduce its PDK1 inhibition and enhance its PAK inhibition. These alterations resulted in a panel of 17 compounds, among them cpd8 (PI-8) and cpd15 (PI-15) (26). Two compounds,

cpd4 and cpd15, reduced cell viability and cell migration in thyroid cancer cells, and constitutively active PAK1 rescued the anti-migration effect in thyroid cancer cells indicating that both compounds inhibit PAK activation (26). These same studies confirmed that both compounds decreased PAK phosphorylation.

Since PAK regulates signaling pathways that promote cell survival and proliferation in VS, we hypothesized that pharmacological inhibition of PAK will induce cell death in vestibular schwannomas (VS) and meningiomas; therefore, the purpose of this study is to determine the effect of the new small molecules, PI-8 and PI-15, in vestibular schwannoma and meningioma growth. Our present work reports the mechanism of action of PI-8 and PI-15 in vestibular schwannoma and meningioma growth.

Materials and Methods

Cell Culture and Drug Treatment

Cell Lines—The BenMen1 (BM) benign meningioma cell line was provided by Dr. Long-Sheng Chang (The Ohio State University; Columbus, Ohio) and has been described previously (27). BenMen1 cells have a mutation in exon 7 of the NF2 gene, which causes a premature stop codon and therefore a short transcript (28). The HEI193 benign schwannoma cell line was kindly provided by Dr. Marco Giovannini (The House Research Institute, Los Angeles, California). HEI193 cells have NF2 mutation causing a splicing defect in the NF2 transcript, which still allows expressing moderately the active growth suppressive function of merlin (29).

Primary Cells—Primary cells were isolated from tumors of sporadic vestibular schwannoma and meningioma patients under the University of Arizona IRB approval numbers 11-0643-01 and 11-0773-01. VS and meningioma primary cells were identified with S100 and Epithelial Membrane Antigen (EMA) markers, respectively. All cell lines and primary human cells were grown in Dulbecco's modified eagle medium (CellGro) with 10% FBS (Fetal Bovine Serum) (Sigma).

Drugs—PI-8 and PI-15 were developed and provided by Dr. Ching-Shih Chen (The Ohio State University, Columbus, Ohio). Both compounds were dissolved in DMSO (Dimethyl sulfoxide) and stocks of 1mM diluted in DMEM1X were used for *in vitro* experiments.

MTT Assays

Cells were seeded in 96-well plates and treated with varying concentrations of PI-8 or PI-15 (0–5 μ M) for 72hrs. Compound concentrations were tested in replicas of 4 wells and the experiments were repeated 4 times. Cell viability was determined by thiazolyl blue tetrazolium bromide assay (25) and the 50% inhibitory concentration (IC₅₀) was calculated.

Immunoblots

Sub-confluent cells were treated with various concentrations of PI-8 and PI-15 for 24–72 hours. They were then harvested and lysed in cold SDS (Sodium dodecyl sulfate) lysis buffer supplemented with protease inhibitor cocktail (Sigma). Equal amounts of lysate protein

(25µg) were first electrophoresed on TRIS-HEPES NH 4–20% gels (Thermo Scientific, Waltham, MA) and then transferred to PVDF (Polyvinylidene difluoride, Millipore, Billerica, MA) membranes. Membranes were then probed with a variety of antibodies including phospho-PAK S144 (Cell Signaling Technologies), phospho-PAK T423 (Pierce), PAK1/2/3 (Cell Signaling Technologies), phospho-AKT S473 and T308 (Cell Signaling Technologies) as well as total AKT (Pierce).

TUNEL Apoptosis Assays

BenMen1 and HEI193 were plated and treated with 2.5 and 5 µM of PI-8 and PI-15 for 72 hours, untreated (negative control), and (a positive control) DNase I for 30 min. Cells were then fixed with 4 % paraformaldehyde, permeabilized with 0.25% Triton-X 100, and probed using the Click-iT® TUNEL Alexa Fluor® Imaging Assay (Invitrogen, Carlsbad, CA). Fixed and stained cells were then examined using a fluorescence deconvolution light microscope. Gray scale images were captured due to improved contrast/visualization of individual cells.

Immunocytochemistry

HEI193 and BenMen1 cells seeded at 2.5×10^4 cells/glass coverslips in 12-well plates were treated with 2.5 and 5 µM of IC50 and 2X IC50 of PI-8 and PI-15 for 24, 48 and 72 hours at 37° C, 5% CO₂. Cells were fixed with 4% paraformaldehyde, permeabilized with PBDT (0.3% Sodium deoxycholate, 0.3% TX-100/PBS1X) for 30 min and blocked with 3% BSA/ 0.1% TX100 for 1hour. Primary antibody incubation was overnight at 4° C and secondary antibody for 2 hours at room temperature. Microscopic analysis and digital images were taken with the DeltaVision Deconvolution microscope (Applied Precision, Pittsburgh, PA).

Results

PI-8 and PI-15 inhibit cell viability in vestibular schwannoma and meningioma cells at low micromolar concentrations

To determine the effect of PI-8 and PI-15 in VS, meningioma cell lines, and primary cells, cell viability at concentrations 0 to 5µM for 72hours was assessed by MTT assay. PI-8 inhibited cell viability in a dose dependent manner in cell lines, BenMen1 and HEI193 at IC₅₀ concentrations 1.24µM and 3.1µM respectively (Fig. 1A). The IC₅₀ concentration of PI-15 for BenMen1 was 0.65µM and for HEI193 was 1.8 µM (Fig. 1B). Primary meningioma and schwannoma cells were treated with either PI-8 or PI-15 at 0 to 5 µM concentrations for 72 hours. PI-8 inhibited cell viability of both cell types at IC₅₀ concentrations, 3.73 µM for primary meningioma cells and 3µM for primary schwannoma cells (Fig. 1C). The IC₅₀ concentration of PI-15 in primary vestibular schwannoma cells was 2.1 µM and for primary meningioma was 2.2 µM (Fig. 1D). Phase contrast visualization of cells undergoing treatment indicated that both compounds decreased the total number of cells in a dose dependent manner (data not shown). These data indicates that PI-15 is more potent treatment for the cell lines and primary tumor cells than PI-8.

PI-8 and PI-15 induces apoptosis in BenMen1 cells but not in HEI193 cells

To determine if the drop in cell viability was due to inhibition of the cell cycle by both PAK inhibitors, flow cytometry studies were performed in triplicates at 48 and 72 hours. These studies show that both inhibitors did not affect cell cycle in VS and meningioma cell lines (data not shown), indicating that both inhibitors induce cell death as their mechanism of action. To determine if PI-8 and PI-15 induced apoptosis, TUNEL assays were performed. These results showed that both PAK inhibitors induce apoptosis in BenMen1 (Fig. 2A) but not in HEI193 (Fig. 2B). PI-15 was more potent than PI-8 in inducing apoptosis in BenMen1 cells. Further confirmation of this data by western blots shows that PI-15 induces cleavage of the well-known apoptosis initiator and executioner caspases (caspase-9 and caspase-3, respectively) (30) at 2.5 μ M when compared to the control (Fig. 2C). Total caspase-3 decreases at 2.5 μ M correlating with the presence of cleaved fragments (Fig. 2C). This data confirms that PI-15 induces apoptosis in BenMen1 cells.

PI-8 and PI-15 mechanism of cell death is mitotic catastrophe in vestibular schwannoma cell line HEI193 cells

Mitotic catastrophe is known as an oncosuppressive mechanism that senses defective mitotic cells driving them to death by apoptosis, necrosis, and senescence to prevent their proliferation (31–33). Studies done by Hebert et al. have shown that merlin plays an important role in positioning the centrosome during cell division (34), and in our previous immunocytochemistry studies on merlin nuclear partners, we observed that phospho-merlin (phospho serine 518) is localized to the centrosomes of dividing HEI193 and BenMen1 cells. In addition, dividing cells showed features of mitotic catastrophe, such as, lagging chromosomes at anaphase, macronuclei and micronuclei, and over duplication of centrosomes (data not shown). These results indicated that mitotic catastrophe signaling would be a target to induce cell death in HEI193 and BenMen1 cells. Furthermore, several reports have demonstrated that many cancer drugs, such as the microtubule targeting agents (MTAs) and centrosome cycle disruption drugs induce cell death by mitotic catastrophe (32, 35–37), backing up our idea. Therefore, since HEI193 and BenMen1 cells showed features of mitotic catastrophe, we thought that PI-8 and PI-15 may target the mitotic catastrophe signaling to induce cell death.

Moreover, cancer studies have shown that anticancer drugs induce high levels of the autophagy marker ATG5, which induces mitotic catastrophe when translocated into the nucleus, and when localized to the cytoplasm induces autophagy (38, 39). Therefore, the localization and levels of ATG5 and phospho-merlin were assessed by immunocytochemistry in PI-8 and PI-15 treated cells versus the non-treated. This data showed that in HEI193 cells, both PAK inhibitors induced high ATG5 expression levels localized to the nuclei and autophagosomes in the cytoplasm (Figure 3). Furthermore, in agreement with ATG5 localization in the nucleus, PI-8 and PI-15 induced many mitotic catastrophe features, such as over duplication of chromosomes, macronuclei and micronuclei in the majority of cells when compared to the controls (Figure 3). However, very few PI-8 treated BenMen1 cells show ATG5 expression in the nucleus, and most of ATG5 expression was associated with autophagosomes in the cytoplasm. Low dose of PI-15 induced high ATG5 expression associated with autophagosomes, and high PI-15 dose did not show a

difference when compared to the controls (data not shown). These data show that PI8 and PI-15 decreases cell viability by inducing mitotic catastrophe in HEI193 cells, while indicating that PI-8 induces autophagy in BenMen1 cells.

PI-8 induces autophagy in BenMen1

To corroborate if PI-8 induced autophagy in dose and time dependent manner in BenMen1 cells, we investigated the co-localization of two autophagy markers, ATG5 and LC3B (light chain 3B), by immunocytochemistry. During autophagy, LC3 is cleaved at the carboxy terminus yielding the cytosolic form LC3-I (LC3A), and during autophagy, LC3A is converted to LC3B (LC3II), which becomes associated with autophagosomes (40–43). These data show that increasing ATG5 and LC3B expression levels were co-localized to autophagosomes at 48hrs of treatment when compared to 24 hours of treatment (Figure 4A and 4B). Protein lysates of BenMen1 72 hours treatment with IC50 and 2X IC50 were analyzed for the expression levels of autophagy markers, ATG5, ATG12, LC3A and LC3B by western blots. These data show that PI-8 increases the levels of the autophagy markers when compared to the controls (Fig. 4C). These data correlates with the immunocytochemistry data confirming that PI-8 cell death mechanism of action in BenMen1 cells is autophagy.

PI-8 and PI-15 inhibit PAK Phosphorylation in vestibular schwannoma

To determine if PI-8 and PI-15 inhibit the activation of PAK and AKT in vestibular schwannoma cells, HEI193 cells were treated for 24 hours with different concentrations of PI-8 and PI-15 versus DMSO controls. These data show that phospho-PAK-Ser144 is not affected by PI-8 but its phosphorylation decreases in a dose dependent manner with PI-15 (Fig. 5A). However, PAK-Thr423 phosphorylation decreases with both PAK inhibitors in a dose dependent manner. AKT signaling is a cell survival pathway and activated by PAK, treatment of HEI193 cells with PI-8 does not have effect in the phosphorylation of AKT-Thr308 but decreases slightly AKT-Ser473 phosphorylation in a dose dependent manner. In contrast, PI-15 effect is more potent in reducing the phosphorylation of both AKT-Thr308 and AKT-Ser473 phosphorylation in a dose dependent manner (Fig. 5A). Both PAK inhibitors did not have effect in the total expression of AKT and PAK proteins, 1, 2 and 3 in HEI193 cells. These results indicate that both inhibitors affect the growth of VS through the deactivation of PAK/AKT signaling pathway.

PI-8 and PI-15 inhibits PAK phosphorylation in meningioma cell lines

BenMen1 cells, which substantially grow slower than HEI193 cells, were treated with PI-8 and PI-15 versus the DMSO control for 72 hours. Analysis of the PAK/AKT activation was performed by western blots on the same PAK/AKT phosphorylation sites. These data show that PI-8 inhibited phosphorylation of PAK at Thr423 in a dose dependent manner without affecting phosphorylation status of Ser144 (Fig. 5B). Alternatively, PI-15 inhibited the phosphorylation of both PAK phosphorylation sites, Ser144 and Thr423 in a dose dependent manner (Fig. 5B). PI-8 did not affect the phosphorylation of AKT at serine-473 but slightly decreased threonine-308 phosphorylation while PI-15 decreased the phosphorylation of AKT at both phosphorylation sites, Ser473 and Thr308. Both inhibitors did not affect the total expression of PAK and AKT.

Discussion

Vestibular schwannomas and skull base meningiomas are disease-defining neoplasms in patients with neurofibromatosis type 2 (NF2). Often caused by the bi-allelic loss of the *NF2* gene on chromosome 22, these tumors lack functional Merlin (moesin, ezrin, and radixin-like protein), a tumor suppressor protein (1, 44). VS and most meningiomas are histologically benign; however, they can cause hearing loss, balance problems, tinnitus, facial weakness, hydrocephalus, and even death (45). Current treatment options for patients afflicted with these tumors include craniotomy for surgical excision and/or stereotactic radiation therapy; no medical therapies are FDA-approved for NF2-associated tumors. Therefore, developing non-toxic chemotherapy agents effective against both tumor types is an urgent clinical need.

The NF2 community has identified several key intracellular signaling pathways for drug development efforts worldwide, including PI3K/AKT and PAK (46, 47). AKT, a master serine-threonine kinase downstream from phosphatidylinositol-3-kinases (PI3K), is phosphorylated at threonine 308 by its primary activator, PDK1 (phosphoinositide-dependent kinase 1). A second phosphorylation event at serine-473 is then required for maximal AKT function; this then stimulating downstream effectors like GSK3 β (Glycogen synthase kinase 3 beta), IRS-1 (insulin receptor substrate-1), PDE-3B (phosphodiesterase-3B), BAD (Bcl-2-associated death promoter), caspase 9, Forkhead proteins, NF- κ B (Nuclear Factor Kappa Beta), mTOR (Mammalian Target of Rapamycin), nitric oxide synthase (NOS), Raf protein kinase, BRCA1 (Breast cancer susceptibility gene 1) and others.

Our studies show that the new small molecules PAK inhibitors, PI-8 and PI-15, inhibit PAK phosphorylation in both cell lines. While PI-8 decreases the phosphorylation of PAK at only threonine-423, PI-15 decreases the phosphorylation at both serine-144 and threonine-423 in both cell lines. Deactivation of AKT is accomplished by PI-15 by decreasing the phosphorylation of serine-473 in both cell lines. However, threonine-308 is decreased slightly only in BenMen1. This variance may be important in the difference of type of cell death between BenMen1 and HEI193 cells, PI-15 induces apoptosis only in BenMen1 cells while in HEI193 cells induces mitotic catastrophe. PI-8 induces autophagy in BenMen1 cells and does not inhibit the phosphorylation of AKT at serine-473 in both cell lines but it inhibits threonine-308 phosphorylation only in BenMen1 cells, which indicates a correlation with the high levels of expression of the autophagy markers.

Hebert et al. reported that merlin plays role in positioning the centrosome during interphase (34) suggesting that abnormal merlin function may induce defective mitotic cells by inducing mitotic features (such the ones we report here) that allow the proliferation of these cells. Our data show that PI-8 and PI-15 induce mitotic catastrophe in HEI193 suggesting that these PAK inhibitors facilitate the functioning of molecules involved in the mitotic catastrophe signaling, such as the translocation of ATG5 into the nucleus (38). Cell death by mitotic catastrophe induced by these new PAK inhibitors is a new finding/idea in the field of vestibular schwannomas and meningiomas. However, the genetic make up of these benign tumors due to mutations in the NF2 gene, and NF2 and CHEK2 mutations (in the case of

meningiomas (48)), leading to centrosome amplification, indicate that this mechanism of cell death is consistent with the role that merlin plays on the positioning of the centrosome during interphase. The location of the mutations in the NF2 sequence can also dictate the effect PI-8 and PI-15 in the difference of the doses and effect on cell viability, as well as the type of induced cell death between primary cells and cell lines.

Though to develop these studies we had limitations concerning the availability of more cell lines and primary cells, our data show promising PAK inhibitors that represent putative drugs to develop for the treatment of vestibular schwannoma and meningiomas. Future studies in our lab will focus on evaluating these drugs using cell line xenografts into chicken CAM (Chorioallantoic membrane) Assays.

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Figure 1. PAK Inhibitors decrease cell viability in a dose dependent manner in VS and meningioma cells

A. HEI193 and BenMen1 cell lines decreased 50% viability with PI-8 at 1.24 μM and 3.1 μM respectively. **B.** PI-15 decreased HEI193 and BenMen1 cell viability at IC50 1.8 μM and 0.6 μM respectively. **C.** Primary vestibular schwannoma and primary meningioma cells decreased 50% viability with PI-8 at 3.25 μM and 4.1 μM respectively. **D.** PI-15 IC50 at 2.1 and 2.2 μM decreased cell viability in primary VS and meningioma cells respectively. Error bars represent SEM.

Figure 2. PAK Inhibitors induce apoptosis in BenMen1 but not in HEI193 cells

A. TUNEL: PI-15 (2.5 μ M) induces apoptosis in more BenMen1 cells than PI-8 (2.5 μ M) compared to 0 μ M (Negative Control). Hoechst dye shows nuclear staining of the cell sample in the positive control, negative control, and PI-8 treated cells while the PI-15 treated population of cells is very small. **B.** TUNEL: PI-8 or PI-15 at 2.5 μ M did not induce apoptosis in HEI193 cells when compared to Negative control. In **A** and **B** Positive control conforms cells treated with DNase (Invitrogen, Carlsbad, CA). **C.** BenMen1 treated cells with PI-15 for 72 hours show increase in cleavage of the apoptosis initiator, caspase-9, into 39 and 37 kilodaltons (kDa), and of the executioner caspase-3 into 19 and 17 kDa at 2.5 μ M when compared to the control. Total caspase-3 (35 kDa) decreases in treated cells at 2.5 μ M. GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) is the loading control.

Figure 3. PI-8 and PI-15 induces cell death by mitotic catastrophe in HEI193 cells

A. Control cells show phospho-merlin expression (red). Cell in the middle is bi-nucleated (DAPI: blue). **B.** HEI193 cells treated with 2.5- μ M PI-8 show ATG5 expression (green and white arrows) in the nucleus of many cells. ATG5 expression is associated with autophagosomes in the cytoplasm (blue arrowheads). Phospho-merlin expression is visualized in centrosomes (yellow arrows) holding two nuclei together. **C.** HEI193 cells treated with 5- μ M PI-8. Increased autophagosome formation associated with high levels of ATG5 and nuclear ATG5 is visualized. **D.** 2.5- μ M PI-15 induced high ATG5 expression levels associated with autophagosomes in the cytoplasm and in the nucleus. Many centrosomes are visualized with phospho-merlin expression (yellow arrows). **E.** 5- μ M PI-15 in HEI193 cells show ATG5 expression in the nucleus and associated with autophagosomes. Treatment was for 72 hours. Images were taken using the Delta Vision Deconvolution microscope. 60X.

Figure 4. PI-8 induces cell death by autophagy in BenMen1 cells

A. ATG5 (green) and LC3B (red) expression increases in BenMen1 cells treated with increasing doses of PI-8 at 48 hours. **B.** 24-hours BenMen1 treated cells show ATG5 and LC3B expression increase in a dose dependent manner. Merged: ATG5 and LC3B co-localization (yellow). **C.** Autophagy markers expression increased in BenMen1 cells treated for 72 hours with increasing doses of PI-8.

Figure 5. PI-8 and PI-15 inhibit PAK phosphorylation in vestibular schwannoma and meningioma cells

A. In HEI193 cells, progressive exposure to PI-8 decreased PAK phosphorylation at the threonine phosphorylation site. Progressive exposure to PI-15 decreased PAK phosphorylation at both threonine-423 and serine-144. Total PAK and AKT levels were stable in both treatment groups. At higher doses, PI-15 inhibits AKT phosphorylation at its threonine and serine phosphorylation sites. **B.** Progressive exposure to PI-15 decreases PAK phosphorylation at both phosphorylation sites. Total PAK and AKT levels were stable in both treatment groups. At higher doses, PI-15 inhibits AKT phosphorylation at its threonine and serine phosphorylation site.