PTEN loss mitigates the response of medulloblastoma to Hedgehog pathway inhibition


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Precis

This study offers new insights into the potential efficacy of Hedgehog pathway inhibitors being tested clinically against a common pediatric cancer.

Abstract

Medulloblastoma (MB) is a cancer of the cerebellum, for which there is currently no approved targeted therapy. Recent transcriptomics approaches have demonstrated that MB is comprised of molecularly distinct sub-groups, one of which is characterized by activation of the Hedgehog (Hh) pathway, which in mouse models is sufficient to drive MB development. There is thus considerable interest in targeting the Hh pathway for therapeutic benefit in MB, particularly given the recent approval of the Hh pathway inhibitor vismodegib for metastatic and locally advanced basal cell carcinoma (BCC). Like other molecularly targeted therapies however, there have been reports of acquired resistance to vismodegib, driven by secondary Hh pathway mutations and potentially by activation of the phosphatidylinositol 3-kinase (PI3K) pathway. Given that acquired resistance to vismodegib may occur as a result of inappropriate PI3K pathway activation, we asked if loss of the PI3K pathway regulator, phosphatase and tensin homologue (Pten), which has been reported to occur in patients within the Hh sub-group, would constitute a mechanism of innate resistance to vismodegib in Hh-driven MB. We find that Hh pathway inhibition successfully restrains growth of Pten-deficient MB in this mouse model, but does not drive tumor regression, as it does in Pten-wildtype MB. Combined inhibition of the Hh
and PI3K pathways may lead to superior anti-tumor activity in PTEN-deficient MB in the clinic.
Introduction

MB is the most common malignant pediatric brain tumor, and is the leading cause of cancer-related mortality in childhood. Current available therapy consists of surgery, radiation therapy of the brain and spinal cord, followed by chemotherapy. Unfortunately, this aggressive therapeutic strategy is associated with serious adverse effects, including postoperative mutism, neurocognitive deficits, endocrinopathies, and increased susceptibility to secondary malignancies (1).

Recent transcriptional profiling has demonstrated that MB is comprised of four molecularly distinct sub-groups: the Wnt sub-group, Sonic Hedgehog sub-group, Group 3 and Group 4 (2). The identification of these sub-groups provides a much needed opportunity for the use of molecularly targeted therapeutics in MB, which have the potential to both increase efficacy and reduce side effects when compared to the current standard of care. The Hh sub-group, which makes up approximately 25% of MB cases (3), may be particularly amenable to a targeted therapeutic approach, given the recent FDA approval of the Hh pathway inhibitor, vismodegib (previously known as GDC-0449), in metastatic and locally advanced BCC.

Hh signaling is critical for embryonic development and also plays a limited role in adult homeostasis. In the off-state, the transmembrane receptor Patched (PTCH) suppresses the activity of the GPCR-like molecule Smoothened (SMO). The Hh ligands (Sonic, Indian or Desert Hh) activate the pathway by binding to PTCH, which releases its inhibition over
SMO. SMO in turn signals to the GLI family of transcription factors and thus induces a change in the transcriptional profile of the cell (4). Inappropriate activation of the pathway, most often caused by inactivating mutations in \textit{PTCH}, or activating mutations in \textit{SMO}, is associated with cancer, predominantly BCC and MB (5-8).

Vismodegib binds to, and inhibits SMO, blocking downstream Hh pathway activation. In early-stage clinical studies, a MB patient from the Hh sub-group harboring widespread metastatic disease, exhibited rapid and dramatic tumor regression when treated with vismodegib (9). Unfortunately, the robust initial response was followed by relapse, reminiscent of the acquired resistance seen in the context of other targeted therapies, such as erlotinib (10). Sequence analysis of a tumor biopsy obtained after progression in this patient indentified an amino acid substitution at a conserved residue of SMO. The D473H substitution disrupts the ability of vismodegib to bind SMO, but does not alter the ability of SMO to activate the Hh pathway (11). This observation triggered a series of investigations into potential alternative mechanisms of resistance against vismodegib and sonidegib, another Hh pathway inhibitor currently under clinical development (12). A variety of resistance mechanisms were identified through the use of mouse models, including additional mutations in \textit{Smo}, as well as downstream Hh pathway alterations such as \textit{Gli2} amplification (11, 13, 14). An additional, and unexpected potential mechanism of acquired resistance to sonidegib was the up-regulation of PI3K signaling (13).

The PI3K pathway is a well-established oncogenic pathway that regulates cell growth, proliferation, and survival by relaying growth factor signaling (15). Aberrant activation of
the PI3K pathway occurs in multiple tumor types and is frequently driven by disruptions in the negative pathway regulator PTEN, but can also be driven by gain-of-function mutations in PIK3CA. A recent genomic analysis of MB tumors revealed that of 13 Hh sub-group patients profiled, two had loss of function mutations in PTEN, and another patient had an activating mutation in PIK3CA (16). Of 66 patients profiled from the other sub-groups, none had loss of PTEN, and two had mutations in PIK3CA. Another sequencing study likewise found a number of PTEN mutations in medulloblastoma tumors, one of which co-occurred with a homozygous PTCH mutation (17). In addition to genomic alterations as a mechanism to hyperactivate the PI3K pathway, epigenetic inactivation of PTEN has been reported to occur at a high frequency in MB samples (18). Given that inappropriate activation of PI3K signaling may represent a potential mechanism of acquired resistance to Hh pathway inhibitors, we wanted to determine if pre-existing PTEN mutations, which occur in Hh sub-group patients, would alter the initial response to vismodegib in Hh-driven MB.
Materials and Methods

Mouse models

Math1<sup>CreER<sup> and Pten<sup>pexp mouse strains were both obtained from The Jackson Laboratory (stock numbers 007684 and 004597, respectively), and the Ptc<sup>pexp strain was a kind gift from R. Toftgard and S. Teglund (Karolinska Institutet) (19). All mice were housed and maintained according to the animal use guidelines of Genentech, conforming to California State legal and ethical practices. Cre-dependent recombination was induced by oral gavage of tamoxifen (single dose of 4 mg in sunflower seed oil) to pregnant dams at E14.5.

Tissue analysis

Tumors were harvested from moribund animals and subject either to overnight fixation in 4% paraformaldehyde prior to embedding in paraffin, or to lysis in CellLytic MT Lysis reagent (Sigma). Immunohistochemistry (IHC), immunofluorescence (IF), and western blotting (WB) were carried out according to standard procedures. Antibodies used were as follows: mouse anti-NeuN (1:500 for IF and 1:1000 for WB; Millipore, # MAB377), rabbit anti-Ki67 (1:300, Thermo Scientific, # RM9106), rabbit anti-pAKT S473 (1:500 for IHC and 1:1000 for WB, Cell Signaling Technology, # 4060), rabbit anti-pS6 (1:1000, Cell Signaling Technology, # 4858), rabbit anti-PTEN (1:100 for IHC and 1:1000 for WB, Cell Signaling Technology, # 9188) and rabbit anti-cleaved caspase 3 (1:100 for IHC, Cell Signaling Technology, # 9661). The DAKO Envision+ HRP (DAB) system was used to detect primary
antibodies for IHC. Images were acquired using a Zeiss Axioskop2 plus microscope fitted with an AxioCam HRC, or a Leica DMI4000B fluorescence microscope. Adobe Photoshop CS3 was used to process images, and Fiji was used to quantify cleaved caspase 3 staining.

**Generation of MB allografts and drug treatments**

To enable an assessment of the sensitivity of these MB models to inhibition of the Hh and PI3K pathways, tumor growth was relocated subcutaneously via grafting. The cerebella of mice displaying symptoms associated with MB were harvested and prepared for subcutaneous inoculation into the right lateral thorax of female CD1-nude (CRL) mice aged at least 6 weeks, to establish passage 1. Allografts were generated via inoculation of a single cell suspension in 100 µl Neuralbasal Medium (Invitrogen) (PM model, derived from $P_{tch}^{lox/p; Math1^{CreER}}$ mice), or small tumor fragments (PPM model, derived from $P_{tch}^{lox/p; Pten^{lox/p}; Math1^{CreER}}$ mice). Serial in vivo propagation of the PPM model was repeated to generate sufficient tumor-bearing animals for drug treatment. Tumors were measured with calipers, and tumor volumes calculated using the formula $v = 0.5 \times \text{length} \times \text{width}^2$, where length and width represent perpendicular tumor diameters. As tumors reached 100 mm$^3$ tumor-bearing animals were separated into groups of similarly sized tumors and drug administration was initiated.

Compounds were formulated in MCT and mice were administered either vehicle or drug once daily (QD) by oral gavage; vismodegib and GNE-317 treatments were separated by 4 hours. To generate samples for pharmacodynamic analysis (qPCR and WB), tumor-bearing
mice were treated for 3 consecutive days, and tumors were harvested 4 hours after the final dose.

**Statistical methods**

A mixed modeling approach was used to analyze the repeated measurement of tumor volumes from the same animals over time (20). This approach addresses both repeated measurements and modest dropouts before study end. Restricted cubic splines were used to fit a non-linear profile to the time courses of \( \log_2(\text{tumor volume}) \) in each group. Fitting was done via a linear mixed effects model, using the R package nlme, version 3.1.97 in R version 2.12.0 (R Development Core Team 2008; R Foundation for Statistical Computing; Vienna, Austria).

Tumor growth inhibition as a percentage of vehicle control (%TGI) was calculated as the percentage of the area under the fitted tumor volume-time curve (AUC) on the linear scale for the respective treatment group per day in relation to the starting volume on Day 0 (baseline) and in relation to the vehicle. As such, a TGI value of 100% indicates tumor stasis; a TGI value of \( >1\% \) but \( <100\% \) indicates tumor growth delay; and a TGI value of \( >100\% \) indicates tumor regression and would be associated with a negative AUC.

To determine uncertainty intervals (UIs) for %TGI, the fitted curve and the fitted covariance matrix were used to generate a random sample as an approximation to the distribution of %TGI. The random sample is composed of 1000 simulated realizations of the fitted-mixed model, in which the %TGI is recalculated for each realization. Our
reported UI is the value for which 95% of the time the recalculated values of %TGI will fall in this region given the fitted model. The 2.5 and 97.5 percentiles of the simulated distribution were used as the upper and lower UIs, respectively. All TGI values were calculated at day 13 of the experiment, to allow for direct comparison across the two models; TGI cannot be calculated beyond day 13 in the PPM model as control/vehicle-treated tumor-bearing animals were euthanized before the end of the study, because tumor volumes exceeded 2000 mm³.

Results

Loss of PTEN accelerates the onset of morbidity in a mouse model of Hh-driven MB

An earlier study showed that deletion of Ptch in cerebellar granule neural precursor cells (CGNPs), which was achieved by crossing Math1CreER mice to PtchloxP/loxP mice, resulted in the development of MB, with 100% penetrance (21). For our studies, we combined this model with the previously described PtenloxP/loxP mouse allele to generate PtchloxP/loxP; PtenloxP/loxP; Math1CreER compound mutants (22). Similar to the previous report, PtchloxP/loxP; PtenloxP/loxP; Math1CreER (hereafter referred to as PM) mice (induced with tamoxifen at E14.5) display signs of illness during early adulthood (6 to 10 weeks), which include a domed head and abnormal gait. Strikingly, loss of Pten in PtchloxP/loxP; PtenloxP/loxP; Math1CreER (referred to as PPM) mice dramatically accelerates disease, with animals as young as 3 weeks of age presenting with ataxia; the median survival is 9.3 weeks in PM versus 4.0 weeks in PPM mice. PtchloxP/loxP; Ptenwt/loxP; Math1CreER (PPwt/loxPM) littermates have an intermediate
survival, with a median of 7.4 weeks (Fig. 1A). Importantly, loss of Pten alone in this model is not sufficient to initiate observable disease, as $Ptch^{wt/wt}; Pten^{loxp/loxp}; Math1^{CreER}$ mice survive long-term with no symptoms.

Analysis of brain samples from PM and PPM mice revealed very large tumors within the cerebellum, which disrupt the normal cerebellar architecture (Fig. 1B, C, D). Histologically, tumors from PM mice resemble classic MB, which is the histological sub-type that predominates amongst human patients from the Hh sub-group; these are frequently described as "small round blue-cell" tumors (Fig1 C). In contrast, tumors from PPM mice have a markedly different appearance, with a histological phenotype analogous to medulloblastoma with extensive nodularity (MBEN), which in humans, is a histological sub-type thought to be restricted to Hh sub-group patients (3). Tumors from $PP^{wt/loxpM}$ mice are histologically indistinguishable from PPM tumors, with a MBEN appearance (Suppl. Fig. 1). The cerebella of $Ptch^{wt/wt}; Pten^{loxp/loxp}; Math1^{CreER}$ mice lack tumors, however, we did detect large ectopic cells both external to, and within the molecular layer (Suppl. Fig. 2).

**Activation of PI3K signaling drives MB cells towards a more differentiated phenotype**

Immunohistochemical analysis revealed additional differences between PM and PPM tumors. PM tumors are largely made up of undifferentiated cells, positive for Ki67 and negative for the neuronal marker NeuN (Fig. 2A, see also Suppl. Fig. 3 for phospho-histone H3 IHC). Somewhat counter-intuitively, the more aggressive PPM tumors have a high
content of differentiated cells, positive for NeuN and negative for Ki67, with Ki67+ cells occurring sporadically within the tumor mass. Strikingly, in MB tumors that are either $Pten^{loxp/loxp}$ or $Pten^{loxp/wt}$, in addition to scattered Ki67 positive cells, we also find regions of Ki67+/NeuN- cells that occur as “stripes” (Fig. 2B, and Suppl. Fig. 3 for pH3 IHC). These proliferative stripes appear to localize to perivascular regions (see Fig. 2C inset), an observation that was confirmed by EdU/CD31 double-staining, in tumors harvested 2 hours after administration of the thymine analog (Fig. 2D). PM tumors have a relatively high level of baseline apoptosis, as revealed by the presence of cleaved caspase 3 (CC3), which appeared to be markedly reduced in PPM tumors (Fig. 2E, F). We used image analysis to quantify this apparent difference, taking into account the observation that in any field of view there appear to be more cells in the PM model than in the PPM model, and found that indeed, apoptosis is significantly suppressed in the PPM model (Fig. 2G, see also Suppl. Fig. 4 for an illustration how the image analysis was conducted).

**Loss of PTEN in Hh-driven MB alters the response of tumors to vismodegib**

The early onset of tumors in the PM and PPM models, the very rapid onset of morbidity of the PPM model in particular, and the lack of normal cerebellar tissue in this model, which would be required to maintain cerebellar functionality after targeting the tumor, prevented us from performing a vismodegib intervention study in the autochthonous setting. Fortunately however, the value and relevance of allograft studies in the context of MB have previously been well established (11, 13, 14). Therefore, to evaluate the response of PM and PPM tumors to vismodegib, we generated allograft models; tumors harvested from PM
and PPM animals were passaged subcutaneously in nude mice. This allowed tracking of tumor volumes over time, in response to various doses of drug (see also Methods). Although the histological characteristics distinguishing primary PM and PPM tumors do not appear to be strictly maintained in the allograft setting, key features, such as PTEN status and pAKT levels (Fig. 3A, B), are maintained. Note the loss of PTEN in PPM tumor cells, but not in stromal cells, consistent with the restriction of cre-recombinase activity to \textit{Math1}-expressing neural progenitors. As expected, loss of PTEN is associated with robust up-regulation of pAKT. In addition, PPM allografts appear to retain a somewhat more differentiated phenotype when compared to PM allografts, with apparent increases in NeuN levels (Fig. 3C), though the differentiation phenotype is less dramatic in the allografts when compared to the primary tumor.

Oral gavage of mice with 3 mg/kg vismodegib QD has little effect on allograft growth, in either model, despite having a modest effect on Hh pathway activity (Fig. 4A, B, D). This finding, that \textasciitilde50\% Hh pathway inhibition is not sufficient to drive a tumor response, is consistent with the recent demonstration that sustained and robust inhibition of the Hh pathway (>80\%) is required for a meaningful anti-tumor effect (23). In contrast, 30 mg/kg vismodegib QD is sufficient to cause a meaningful growth delay in both models, with a mean tumor growth inhibition (TGI) of 90\% in the PM model, and 70\% in the PPM model (Fig. 4A, B, C).

Interestingly, the response to vismodegib appears to diverge between the models at higher doses of vismodegib. In the PM model, vismodegib dosed at 60 mg/kg and 90 mg/kg QD
results in rapid and robust tumor regression, with 149% and 159% mean TGI respectively; tumors continued to regress beyond day 13, and were barely detectable at day 21, when the study ended (Fig. 4A, C). In contrast, PPM tumors had not regressed by day 13, on either 60 mg/kg or 90 mg/kg vismodegib, with a mean TGI of less than 100% in both cases (87% and 90%); rather, these doses achieved tumor stasis by the end of the study, despite a comparable level of Hh pathway inhibition (Fig. 4D).

**Combined PI3K and Hh pathway inhibition drives tumor regression in the PPM model**

GNE-317 is a PI3K/mTOR pathway inhibitor that was specifically designed to cross the blood-brain barrier, with the treatment of PI3K pathway-driven glioblastoma as the primary objective (24). We made use of this previously described compound to ask if inhibition of the Hh pathway in combination with inhibition of PI3K signaling, would improve on vismodegib as a single agent. GNE-317 when dosed at 30 mg/kg QD successfully attenuates PI3K signaling in the allograft setting, as measured by pS6 levels (Fig. 5A), and by pAKT levels (Suppl. Fig. 5), but does not alter levels of Hh pathway activity (Fig. 4D). Likewise, 90 mg/kg vismodegib does not influence activation of the PI3K pathway in this setting (Suppl. Fig. 5). GNE-317, as a single agent, achieved modest inhibition of tumor growth in the PM model, and a more meaningful inhibition of growth in the PPM model (mean TGI 37% and 66% respectively) (Fig. 5B, C, D). In the PPM model, while vismodegib treatment alone did not drive regression at any concentration tested, GNE-317 treatment, when combined with either 60 mg/kg or 90 mg/kg vismodegib,
resulted in tumor regression (Fig. 5B, D). At 60 mg/kg vismodegib plus GNE-317, mean tumor volume at the study start was 232 mm$^3$, which regressed to 152 mm$^3$ on day 13 and 100 mm$^3$ by the study endpoint. At 90 mg/kg vismodegib plus GNE-317, mean tumor volume at the study start was 244 mm$^3$, which regressed to 102 mm$^3$ on day 13 and 80 mm$^3$ by the study end. It is also worth noting that the regression driven by the combination of GNE-317 plus 90 mg/kg vismodegib is highly consistent across individual tumors/animals (Fig. 5D).

**Discussion**

Inherited mutations in PI3K signaling components are associated with human syndromes that include neurological abnormalities, such as Lhermitte-Duclos disease, indicating that this pathway is important in normal neural development and/or function (25). Indeed, two independent mouse studies described the use of $Gfap^{Cre}$ to delete $Pten$ in the brain, and showed that this led to lethal neurological defects including seizures and ataxia, which were coupled to an enlargement of the cerebellum (25, 26). Histological analysis revealed the presence of ectopic, enlarged cells at the pial surface and within the molecular layer of the cerebellum, which was attributed to a migration defect in mutant cells. Histologically, this phenomenon is very similar to the phenotype seen in $Ptch^{wt/wt}; Pten^{loxp/loxp}; Math1^{CreER}$ mice, though the mice described here do not display any overt clinical symptoms, potentially due to incomplete deletion of PTEN driven by the $Math1^{CreER}$ (see Suppl. Fig. 2). A more recent study demonstrated that postnatal deletion of PTEN in migrating neuroblasts in the rostral migratory stream resulted in ectopic positioning and altered...
morphology of neurons. The authors argue that the migration defect associated with PTEN loss was likely secondary to precocious differentiation, rather than a defect in the mechanics of directional migration (27). The highly differentiated nature of the tumors in PPM mice described here, as well as NeuN expression in ectopic cells in the cerebella of Ptc\textsuperscript{wt/wt}; Pte\textsuperscript{loxp/loxp}; Math\textsuperscript{1CreER} mice, supports the notion that loss of PTEN may drive precocious/premature differentiation in neurons, while the presence of PTEN-deficient neurons within the inner granule layer argues against a migration defect of these cells. Perhaps most relevant, the RCAS/tv-a system, which allows postnatal gene transfer in a cell type specific manner, was used to generate MB by targeting SHH to nestin-expressing neural stem cells, either in the presence or absence of PTEN (28). Intriguingly, PTEN deficient tumors were described as having MBEN histology, with high levels of NeuN, very similar to the PPM model described here. Moreover, the perivascular niche of these tumors was described as being highly proliferative, “in fact the only proliferative region in the tumors”, again, very similar to the PPM model described here. We propose that these proliferative cells in the perivascular niche are major contributors to tumor growth. A different study, using a constitutively active mutant of Smo, Smo\textsuperscript{A1}, to induce MB in Pte\textsuperscript{+/−} mice likewise demonstrated that activation of PI3K signaling accelerated tumorigenesis while driving a switch from classic to MBEN histology, and concomitantly increased neuronal differentiation (29). Notably though, while there is a clear relationship between hyperactivation of the PI3K pathway and MBEN histology that is recapitulated in a number of different mouse studies, it remains to be established if the same relationship exists in human patients.
That activation of PI3K signaling can simultaneously promote differentiation while also accelerating tumorigenesis is somewhat surprising; differentiated tumors are generally associated with slower growth and a better prognosis. Intriguingly though, blocking apoptosis in a SmoA1 model of MB, by deleting pro-apoptotic Bax, resulted in a very similar phenotype: a paradoxical increase in differentiation coupled to acceleration of disease (30). Strikingly, SmoA1; Bax−/− tumors appear indistinguishable from the PPM tumors described here (see Figure 5 Garcia et al.). The authors propose a model in which MB cells face a cell fate choice between apoptosis and differentiation; those cells that are competent to undergo apoptosis will do so and will thus be lost from the tumor, resulting in tumor turnover. In contrast, cells incapable of launching an apoptotic program will adopt a terminally differentiated fate, leading to the accumulation of differentiated cells. The differentiated, non-apoptotic, and aggressive phenotype of the PPM model described here, together with the established role of PI3K signaling in promoting cell survival is in line with this fate choice model.

Despite the profound changes that occur in PTEN deficient tumors with respect to PTEN wildtype tumors, PPM allografts are responsive to vismodegib: inhibition of the Hh pathway restrains tumor growth and results in stasis, suggesting that even in the context of PTEN loss, tumors remain highly dependent on Hh pathway activity for growth. Truly resistant tumors, for example allografts that harbor the mouse equivalent of the patient-derived SMO D473H mutation described above, continue to grow robustly in the presence of 75 mg/kg vismodegib (11). Importantly then, while hyperactivation of PI3K signaling prevents regression of tumors in response to Hh pathway inhibition, it does not confer
bona fide resistance. Nevertheless, the distinction between stasis and regression is of critical importance, and raises a key question: how is regression driven by Hh pathway inhibition in the PM model, and how is this altered by the loss of Pten? Another open question relates to the durability of the drug response in these models; specifically how the durability is influenced by the lack of regression in vismodegib-treated Pten mutant tumors. It is likely that in the absence of complete regression, discontinuation of drug treatment will lead to immediate re-initiation of tumor growth, as occurs in Ptc1+/−; p53−/− allograft tumors (11).

Our findings have significant implications for the clinical development of Hh pathway inhibitors in MB, which are currently ongoing [see clinicaltrials.gov, and references (31-33) for descriptions of Hh pathway inhibitors currently under clinical development]. In the case of BCC, for which vismodegib is now approved, the vast majority of tumors display alterations in the Hh pathway, and there is no current requirement for the stratification of patients. However, the additional genomic complexity seen in MB patients means that it will be critical to pre-select the ~25% of patients who are defined as the Hh sub-group. Based on the data described here, we now argue that it will also be important to monitor PI3K pathway status during patient selection, particularly in cases where a positive clinical trial outcome is defined by regression, rather than stasis. The diagnostic strategy, which will enable the selection of patients who may respond to Hh pathway inhibitors as a single agent, versus those who might benefit from a combined regime of a Hh pathway inhibitor plus a brain penetrant PI3K inhibitor, will require careful consideration, given that PI3K pathway alterations in MB have been reported not only at the genomic, but also at the
epigenetic level (16, 18). It will also be critical to determine if and how other alterations that have been reported to occur in the Hh sub-group, such as \textit{N-myc} amplification, will alter the response of Hh-driven MB to Hh pathway inhibition.
References


Figure Legends

**Figure 1.** Loss of *Pten* in Hh driven MB accelerates tumor onset and alters tumor histology. A, Animals displaying hydrocephalus with clear ataxia were humanely euthanized, and age at sacrifice was used to generate a Kaplan-Meier survival curve. *Ptch*\(^{wt/wt}\); *Pten*\(^{loxp/loxp}\); *Math1*\(^{CreER}\) mice do not succumb to disease and are still alive a year after birth; those with loss of *Ptch*, including *Ptch*\(^{loxp/loxp}\); *Pten*\(^{wt/wt}\); *Math1*\(^{CreER}\) (PM), *Ptch*\(^{loxp/loxp}\); *Pten*\(^{wt/loxp}\); *Math1*\(^{CreER}\) and *Ptch*\(^{loxp/loxp}\); *Pten*\(^{loxp/loxp}\); *Math1*\(^{CreER}\) (PPM), all show severe clinical symptoms before 20 weeks of age. B, C, D, Hematoxylin and eosin (H&E) staining of a normal cerebellum (B), and cerebellar tumors from PM (C), and PPM (D) mice. Note the distinct appearance of the PM tumor bulk, with very tightly packed nuclei and almost no eosinophilic material, versus the PPM tumor with less densely packed nuclei separated by eosinophilic tissue. Granule cells in the presumptive inner granule layer (IGL) are outlined. Scale bars represent 1 mm in the low magnification images, and 100 µm in the high magnification images.

**Figure 2.** Activation of the PI3K pathway promotes differentiation in the context of Hh driven MB. A, PM tumors have a consistent pattern of Ki67 positivity and lack of differentiation throughout the tumor bulk. NeuN is captured in the green channel, and Ki67 in red. Differentiated granule cells of the IGL are outlined, and an asterisk indicates the tumor bulk. B, PPM tumors, as well as those from *Ptch*\(^{loxp/loxp}\); *Pten*\(^{wt/loxp}\); *Math1*\(^{CreER}\) mice (C) stain positive for NeuN throughout the tumor bulk, with Ki67 positivity occurring sporadically within the tumor bulk and along putative blood vessels. Note the presence of
green, auto-fluorescent red blood cells within the proliferative stripe, highlighted in the inset using a white arrow. D, EdU, an analog of BrdU, was administered to $Ptch^{loxp/loxp}$; $Pten^{wt/loxp}$; $Math1^{CreER}$ animals 2 hours prior to harvest, to enable the identification of actively cycling cells. EdU positive cells (green channel) appear to be preferentially clustered near vessels, which stain positive for CD31 (red channel). E, IHC for cleaved caspase 3 (CC3) in a tumor section from a PM mouse, and F, from a PPM mouse. G, Quantification of CC3 staining in PM and PPM tumor sections, individual dots represent % CC3 positive area/total nuclear area within a single image, 7 to 8 images were analyzed per animal ($n = 3$, $p < 0.0001$). Error bars show s.e.m. Scale bars represent 100 µm.

**Figure 3.** PM and PPM allografts reflect key features of the primary tumors. A, H&E staining of PM and PPM allograft models. B, IHC for PTEN and pAKT in primary and allograft models. C, Western blot analysis of lysates generated from 3 vehicle-treated PM, and PPM allografts. Note that NeuN is known to occur as multiple isoforms of distinct molecular weights (34). Scale bars represent 100 µm.

**Figure 4.** Hedgehog pathway inhibition causes robust regression in the PM allograft model, and tumor stasis in the PPM model. A, Dose response of vismodegib on PM tumor growth. Doses of vismodegib are displayed on the top horizontal grey bar, as mg/kg. Grey lines show tumor volume over time for individual animals (1 tumor per animal). The dashed blue lines show the tumor growth fit for vehicle-treated tumors, while the black line shows tumor growth fit for tumors at the indicated doses of vismodegib. Solid red lines indicate tumor volume traces for animals that were euthanized prior to study end due to large
tumors. B, Tumor volume plots from PPM animals at various doses of vismodegib, as above. C, Tumor growth inhibition (%) driven by indicated doses of vismodegib at day 13 of the experiment, compared to vehicle-treated control tumors. D, Expression analysis (qPCR) of the Hh target gene, Hedgehog-interacting protein (HIP), in PM and PPM samples treated as indicated, normalized against expression of the housekeeping gene Rpl19, with MCT treated samples being set to 100%, n = 3. GNE-317 is a PI3K/mTOR pathway inhibitor (discussed below). Note that GNE-317 does not suppress Hh pathway activation. Error bars show s.e.m.

**Figure 5.** A combination approach results in regression of PPM tumors. A, Western blot analysis of allograft lysates treated as shown, demonstrating attenuation of PI3K pathway activity by GNE-317. B, TGI driven by a fixed dose of 30 mg/kg GNE-317 plus indicated doses of vismodegib at day 13 of the experiment, compared to vehicle-treated control tumors. C, Individual tumor volume plots from PM allografts. Doses of vismodegib (mg/kg) are shown across the top grey bar, with a fixed dose of the PI3K pathway inhibitor. D, Individual tumor volume traces from PPM allografts, as above.
A

% survival

0  20  40  60  80  100

Age (weeks)

Ptch$^{wt/wt}$ Pten$^{loxp/loxp}$ (n = 11)
Ptch$^{loxp/loxp}$ Pten$^{wt/wt}$ (n = 14)
Ptch$^{loxp/loxp}$ Pten$^{wt/loxp}$ (n = 17)
Ptch$^{loxp/loxp}$ Pten$^{loxp/loxp}$ (n = 8)

B

Wildtype

C

PM

D

PPM

Metcalfe et al. Figure 1
Figure 4

(A) Tumor Volume (mm³, log2) over time for different treatment groups.

(B) Tumor Volume (mm³, log2) over time for different treatment groups.

(C) Table showing % TGI at day 13 (lower, upper) for different doses of Vismodegib.

(D) Graph showing Relative Expression Level (2^-ΔCt) HIF vs RPL19 for different treatments.

- **Vismodegib (mg/kg)**: 0, 3, 30, 60, 90
- **% TGI at day 13 (lower, upper)**:
  - **0**: 0 (0, 0)
  - **3**: 11 (0, 22)
  - **30**: 90 (52, 115)
  - **60**: 149 (124, 188)
  - **90**: 159 (133, 202)
  - **Vehicle**: 0 (0, 0)
  - **30mg/kg**: 0 (0, 0)
  - **3mg/kg vis**: 0 (0, 0)
  - **60mg/kg vis**: 0 (0, 0)
  - **90mg/kg vis**: 0 (0, 0)

**PM** stands for Primary Malignant, and **PPM** stands for Primary Progressive.
PTEN loss mitigates the response of medulloblastoma to Hedgehog pathway inhibition

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