Personalizing the treatment of pediatric medulloblastoma: Polo-like kinase PLK1 as a molecular target in high-risk children.

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Precis

Results offer preclinical evidence that a pivotal mitotic kinase may be an excellent target for treating a commonly deadly pediatric tumor, with immediate implications for clinical testing of available inhibitors against this kinase.
Abstract

Medulloblastoma (MB) is the most common malignant brain tumor in children. This disease is heterogeneous and it is comprised of four subtypes of MB (WNT, SHH, Group 3, and Group 4). An immediate goal is to identify novel molecular targets for the most aggressive forms of MB. Polo-like kinase 1 (PLK1) is an oncogenic kinase that controls cell cycle and proliferation making it a strong candidate for MB treatment. In this study, pediatric MBs were subtyped in two patient cohorts (Training cohort; n=65 patients, Validation cohort; n=57 patients) using NanoString nCounter analysis and PLK1 mRNA was assessed. We determined that the SHH and Group 3 subtypes were independently associated with poor outcomes in children as was PLK1 using Cox regression analyses. Further, we screened a library of 129 compounds in clinical trials using a model of pediatric MB and determined that PLK1 inhibitors were the most promising class of agents against the growth of MB. In patient-derived primary MB isolates, the PLK1 small molecule inhibitor BI2536 suppressed the self-renewal of PLK1-high but not PLK1-low expressing cells. PLK1 inhibition prevented MB cell proliferation, self-renewal, cell cycle progression, and induced apoptosis. In contrast, the growth of normal neural stem cells was unaffected by BI2536. Finally, BI2536 extended survival in MB-bearing mice with efficacy comparable to Headstart, a standard-of-care chemotherapy regime. We conclude that patients with MB expressing high levels of PLK1 are at elevated risk. These pre-clinical studies pave the way for improving the treatment of MB through PLK1 inhibition.

Introduction
Medulloblastoma (MB) is the most common malignant pediatric brain tumor. The current treatment for MB entails maximal safe resection, whole brain and spinal cord radiation for children over the age of 3, and aggressive chemotherapy. The advances in medical treatments have improved patient survival from 5% in the 1960’s (1) to >70% for the standard-risk disease (2) (3). Yet the five-year survival rate for the high-risk disease is still dismal (16-70%) (3) and almost all survivors will inevitably suffer from adverse, life-long consequences from treatment. These undesirable effects are attributable to the detrimental impacts that surgical procedures, radiation and chemotherapy have on the developing brain (4). Therefore, there is an imperative need to identify novel therapeutics that could improve the cure rate while avoiding harmful side effects.

Medulloblastoma can be divided into four different molecular subtypes: WNT, Sonic Hedgehog (SHH), Group 3 and Group 4. These subtypes were originally described based on differences in gene expression using cDNA microarrays (5), which were subsequently substantiated by immunohistochemistry (IHC) (5, 6). Microarrays are problematic because they require fresh snap-frozen tissues whereas IHC is hindered by the subjectivity of scoring and differential staining across laboratories. Recently, mRNA based assays have been developed using the Nanostring nCounter system to avoid some of the problems associated with prior methods of classification (5). Advantages of NanoString are: 1) multiple genes are used to distinguish MB subtypes, 2) it is highly quantitative, and 3) it does not require an amplification step allowing for low abundance genes to be detected from formalin-fixed paraffin embedded (FFPE) tissues.

The MB subtypes differ not only in genetic signatures but also in response to clinical therapy (7). In studies of MB where adult and pediatric patients were evaluated collectively, the
WNT molecular subtype was associated with the best prognosis while the Group 3 tumors fair the poorest. The SHH and Group 4 tumors correlate with intermediate outcomes in studies that captured adult and pediatric patients (5, 8). However it is not known whether the risk associated with each subtype holds true when the cohort(s) is solely comprised of pediatric patients.

Extensive insights into the biology of the SHH pathway have spearheaded significant progress into the development of related targeted therapies, notably to Smoothened (SMO) for which there are several open clinical trials (9). There are already reports of acquired resistance due to point mutations in SMO (10), amplification in GLI2 (11) and signaling through the PI3K pathway (12). It is therefore possible that other signal transduction pathways may provide alternative approaches to the management of MB. The identification of targeted therapies for Group 3 and Group 4 tumors also remain a challenge. We provide clinical and pre-clinical evidence suggesting that Polo-Like Kinase 1 (PLK1) is a provocative molecular target for pediatric MB that transcends molecular subtypes.

**Material and Methods**

**Medulloblastoma Patient Cohorts**

Primary MBs were obtained from BC Children’s Hospital in Vancouver (Discovery) and The Hospital for Sick Children in Toronto (Validation). In the Discovery cohort we obtained 75 tumor blocks from patients diagnosed with MB between 1986 and 2012. Samples included both primary and relapse specimens, however only primary samples were assessed in this study. Medical charts were reviewed and pertinent clinicopathologic data was recorded (RR, KO and CF). Patient ages ranged from 3 months to 16.8 years old a review of this cohort is shown in Supplementary Table S1. There were 10 of 75 patients excluded from survival analysis for the
purpose of homogeneity of treatment. 1 patient sample was not of MB tumor tissue, and 9 other patients received treatment regimes conflicting with the standard-of-care. Dr. Michael D. Taylor from The Hospital for SickKids University of Toronto generously provided the Validation cohort of 62 MB (Supplementary Table S2). There were 4 of 62 of these patients excluded from survival analysis for the purpose of homogeneity of treatment. All tumors were subtyped and the data analyzed according to Northcott et al. (13).

NanoString nCounter Gene Expression Profiling

RNA was extracted from three 20μm scrolls of FFPE tissue using Qiagen RNeasy FFPE kit (Valencia, CA, USA). Exactly 250ng of RNA was run for each patient sample and RNA quality was assessed using Nanodrop spectrometry. A 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada) was used to spot check RNA quality in random samples. MB cell lines were grown as tumorspheres, and subsequent mRNA analysis was performed in a similar manner to the FFPE tissues. Analysis using nCounter Gene Expression system was conducted at the Centre for Translational and Applied Genomics (CTAG) (BC Cancer Agency, Vancouver, BC). A custom codeset synthesized by NanoString Technologies (Seattle, WA, USA) was designed which included 22 MB specific subtyping gene probes (13) plus other genes of interest which specifically included PLK1 (NM_005030.3). The recommendations outlined by NanoString Technologies were all followed regarding sample preparation, hybridization, detection, scanning, and data normalization.

Gene Expression and Subtype Assignment Analysis
NanoString gene expression data was analyzed as previously described (8, 13-15). Cell lines (grown as neurospheres) and xenograft tumor tissue was assigned subgroup classification using the 74 patient cohort as a training set. Cutoffs for PLK1 expression were assigned based on z score deviation from the mean expression of the cohort. PLK1 transcript counts below 400 (per 250ng patient RNA) were considered low in expression. Heatmaps were generated using unsupervised hierarchical clustering with average linkage using Cluster version 3.0 and Treeview version 1.60.

**Immunofluorescence**

Immunofluorescence staining was performed on Daoy cells according to the procedure we previously described (16). Primary antibodies include: alpha-tubulin (Abcam, ab18251), Pericentrin (Abcam, ab28144), anti-P-H2AX (Abcam, ab26350), and anti-poly(ADP-ribose) polymerase (Cell Signaling Technology). Images were taken using an Olympus FV10i confocal microscope on 60X magnification.

**Drug Library Screen**

The small-molecule targeted therapeutic agents were synthesized, purity checked and purchased from ChemieTek (Minneapolis, MN, USA). The Daoy cell line was seeded (3,000 cells/well) overnight then treated with 1µM and 10µM for 72 hrs. Cells were fixed in 2% paraformaldehyde and stained with Hoechst 33342 (1µg/mL) (Sigma-Aldrich, Oakville, ON, Canada). Analysis was done with the ArrayScan high-content screening system (HCS; Thermo Fisher Scientific, Pittsburgh, PA, USA).
Cell Culture and Tumorsphere Growth

Daoy cells were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). Primary brain tumor cells were isolated from BTX001 (SHH), BT006 (SHH), BT007 (SHH), BT008 (PNET), BT014 (Group 4), BT274, and BT025 (Group 4) and were grown as neurospheres as previously described (17) using Neurocult media (Stem Cell technologies, Vancouver, BC, Canada) (18). All primary cells were obtained through informed consent in abidance with the respective research ethics board guidelines at British Columbia Children’s Hospital and The Hospital for Sick Children. A single-cell suspension of Daoy and patient-derived primary MB cells (BTX001, BT274, BT014, and BT025) were plated in tumorsphere assays as previously described (18). Tumorspheres, plated in triplicate >50μm were quantified and photomicrographs were taken after 6 days of culture.

Transfection and Western Blotting

Small interfering RNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen, Burlington, ON, Canada) as previously described (19). Immunoblotting was conducted using anti-PLK1 (Sigma-Aldrich), anti-P-CDC25CSer198, anti-P-TCTPSer46, and anti-pan-actin (Cell Signaling Technology, Massachusetts, USA). Band densitometry was measured using ImageJ, v1.46r and normalized to Actin.

Cell Cycle Analysis

Cell cycle analysis was done following 24 hour siRNA or BI2536 treatment using flow cytometry as described by Lee, et al. (2012) (18). Histone H3 phosphorylation was tested as previously described using P-histone H3Ser10 (Cell Signaling Technology) (20).
Annexin V Staining and Quantification of Cell Growth by Hoechst Staining

Daoy cells were treated with 2.5nM BI2536 for 48hrs and stained with Annexin V (Promega, Wisconsin, USA) as previously described (21). To evaluate the effect of PLK1 inhibition on cell growth, Daoy or human neural stem cells (hNSC; H9, hESC-derived, GIBCO, Burlington, ON, Canada) were plated (3,000 cell/well) in 96-well plates also following previously outlined methods (19, 21).

In vivo Evaluation of BI2536 Compared to Chemotherapy

Xenografts from Daoy cell line were injected into the right frontal lobe of NOD-CB17-SCID mouse brains according to Research Ethics Board-approved protocols (n=18). Mice were injected with biological replicates consisting of $10^6$ single-cell suspensions and randomly divided into three treatment groups (n=6) following engraftment of orthotopic tumors: control (0.1N HCl), chemotherapy (vincristine, cisplatin, cyclophosphamide), and treatment (BI2536). Intraperitoneal injections were performed for delivery of all study group agents. Control group consisted of one weekly injection of 0.1N HCl for four weeks. Chemotherapy group consisted of one weekly injection of vincristine (1.05µg/kg) and cisplatin (2.5mg/kg) on day 1 followed by cyclophosphamide (0.0352mg/kg) on day 2 for three weeks. Treatment group consisted of one weekly injection of BI2536 (62.5mg/kg, diluted in 0.1N HCl) for four weeks. The mice were observed until they displayed obvious signs of neurological deficits and appeared unwell. Tumors were removed at the end of the study, formalin-fixed, paraffin embedded, stained with hematoxylin and eosin. The histopathology of the tumors was evaluated by a pediatric neuropathologist (C. Dunham).
Statistical Analysis

All quantitative data presented were analyzed as mean value ± standard error. Principal component analysis (PCA) was conducted for 2D modeling of dimensionality reduction of the 22 subtyping genes (8, 13) using Partek Genomics Suite (Partek, St Louis, MO). For the TMA, clinical survival analysis and animal studies, log-rank analysis was performed on the Kaplan-Meier curve to determine statistical significance of the results. Multivariate survival analysis was conducted using Cox regression proportional hazards and a 95% confidence interval. All survival analysis and Spearman’s Rank correlation test were done using SPSS version 20.0 statistical software (IBM, Chicago, IL, USA). The number of samples used and the respective P-values are listed in the figure legends. The level of significance for the in vitro cell growth/death data was determined by Student’s two-tailed T-test and difference in PLK1 expression between subtypes was assessed using one-way ANOVA (*P value<0.05; **P value<0.01).

Results

MB tumor samples were assigned molecular subtype classification using gene expression data and PAM class predication statistical software (Supplementary Tables S3 and S4). Based on these subtypes, patients with SHH and Group 3 tumors had high probabilities of relapse (Fig. 1A) and death (Fig. 1B). Children with WNT or Group 4 tumors relapsed less frequently and lived longer (Fig. 1A and 1B); this was also seen in a separate validation cohort (Supplementary Fig. S1A and S1B). In the Discovery cohort, a heatmap of the patient gene expression illustrates the subtype distribution and includes the patient tumors from which tissue specimens were collected for primary cell culture (Fig. 1C). These data prompted us to address whether there
may be drugs in clinical trials for adult cancers that would be beneficial for combating SHH type
tumors in children; therefore, we subtyped four MB cell lines (ONS76, UW228, UW426 and
Daoy). Using the 22 established MB subtyping genes from Northcott et al. (2012) (13), each of
these cell lines clustered most similarly to the SHH patient subgroup following both hierarchical
clustering (Fig. 1C and Supplementary Fig. S1C) and PCA analysis (Fig. 1D). This classification
was verified using PAM (Supplementary Table S3; PAM-SHH= 1.05+00) and was confirmed
again using analysis in which the Validation cohort acted as the data training set (data not
shown) (13, 14). Subsequently, we conducted a drug library screen in Daoy cells against 129
drugs, most of which are in clinical trials. To be carried forward, compounds had to meet
specific criteria: 1) \( \geq 70\% \) growth inhibition, 2) activity at 1\( \mu \)M and more so with 10 uM, 3)
potential to cross the blood-brain-barrier, 4) currently in clinical trials, and 5) novelty. Initially,
11/129 compounds partially fulfilled these criteria. Some compounds had shown toxicity in
clinical trials or were previously studied. Therefore, we chose to pursue PLK1 because of its
relative novelty as a target and the 90% growth inhibition efficacy demonstrated by the PLK1
inhibitors group: BI6727, BI2536, and GSK461364 (Fig. 2A).

In patients, PLK1 levels were compared to normal cerebellum (CB). PLK1 mRNA was
higher in the vast majority of MB compared to CB using the nCounter system (\( P<0.001 \)) (Fig.
2B and Supplementary Fig. S2A). There was no significant difference in PLK1 expression
between the four MB subtypes (Supplementary Fig. S2B). The mRNA expression of PLK1 was
associated with higher rates of relapse and poor survival as shown in a Kaplan-Meier univariate
analysis (Fig. 2C). In the Validation cohort, PLK1 mRNA levels were also predicative of
outcome (Fig. 2D). There was no significant difference in the overall survival outcomes or PLK1
expression between the study cohorts (Supplementary Fig. S2C).
Multivariate survival analysis of the Discovery and Validation cohorts are displayed in Table 1 and Supplementary Table S5 respectively. The variables identified as independent factors affecting patient survival include: presence of metastasis (HR= 3.916; 95% CI 1.075-14.265), having a SHH MB (HR= 9.815; 95% CI 1.558-61.843), a Group 3 MB (HR=8.924; 95% CI 1.803-44.170), and expression of PLK1 (HR=7.269; 95% CI 1.464-36.084). Likewise, having SHH MB, Group 3 MB, and expression of PLK1 were also significant in the Validation cohort (Supplementary Table S5). Younger patients often do poorly as protocols for children under the age of three avoid radiation (22). This brings to question whether cases not receiving radiation had even worse outcomes (22). Age and radiation treatment were significantly associated with univariate survival analysis, but were not independent prognostic markers in the multivariate analysis. As well, clinical characteristics such as: sex, extent of resection and chemotherapy were not significant variables associated with survival in the log-Rank test (Table 1). Neither age nor radiation was significant in the Validation cohort (Supplementary Table S5).

Primary patient-derived MB cells were obtained from surgical specimens and grown as tumorspheres (BTX001, BT014, BT016, and BT025). RNA was extracted from cultured tumorspheres and compared to matched FFPE sections from the original tumor whereby their gene expression was characterized using nanoString. Cultured primary cells retained subtype and PLK1 expression patterns of the original tumors. This is exemplified with BTX001, BT016 (Fig. 3A), and BT025 (Supplementary Fig. S3A). Interestingly, PLK1 mRNA was higher in BTX001 compared to CB or hNSC by qRT-PCR (Fig. 3B) and BI2536 BTX001 hindered self-renewal upon serial passaging (Fig. 3C). In contrast, BT014 and BT025 cells were derived from Group 4 tumors that expressed low levels of PLK1 (Fig. 3B and Supplementary Fig. S3B) and they were not responsive to BI2536 (Fig. 3C and Supplementary Fig. S3C). Importantly, the growth effects
of hNSCs were negligible when screened with BI2536 as an in vitro evaluation for the safety (Fig. 3D). Expression of PLK1 mRNA in additional freshly isolated primary specimens (BTX001, BT006, BT007, BT274, BT008) was higher than normal human astrocytes (HA), which is another predominant brain cell type. Daoy PLK1 expression was also comparable to the primary MB freshly isolated from patients (Supplementary Fig. S3D).

PLK1 inhibition with siRNA suppressed the Daoy growth by ~90% in 72hrs (Fig. 4A). Deactivation of PLK1 activity was confirmed by down regulation of phosphorylation of its direct substrate, TCTP by ~80% (Fig. 4A) (23, 24). PLK1 levels expressed in Daoy cells were similar to the levels found in poor prognosis MB patients (Supplementary Fig. S4A). Likewise, PLK1 protein and mRNA expression was detected in two additional MB cell lines ONS76 and UW426 (Supplementary Fig. S4A and S4B), which were equally sensitive to PLK1 inhibition (Supplementary Fig. S4C). As expected, BI2536 suppressed phosphorylation of the known PLK1 substrates TCTP and CDC25C and reduced cell growth with an IC₉₀ of 5nM at 72hrs (Fig. 4B). BI2536 treatment at 24hrs halted the cell cycle and caused G₂/M arrest with observable compromised cell cycle and division as shown by immunofluorescence staining (Fig. 4C and Supplementary Fig. S4D). PLK1 inhibition in Daoy cells resulted in irregular centrosome duplication and separation with many cells appearing polynucleated relative to control treatments (Fig. 4C and Supplementary Fig. S5). Both siPLK1 and BI2536 treatments induced apoptosis at 48hrs (Fig. 4D) and self-renewal was abolished with 10nM BI2536 (Supplementary Fig. S6A).

Daoy and two additional MB cell lines responded to chemotherapeutic agents (vincristine, cisplatin and etoposide) that are used for MB treatment (Fig. 5A and Supplementary Fig. S6B). Therefore, we used an animal model to compare the in vivo efficacy of BI2536 and to conventional chemotherapy using a chemotherapy commonly used in protocol. BI2536 delayed
MB tumor growth and was comparable to standard-of-care chemotherapy protocols (Fig. 5B). It is important to note that the histomorphology of the Daoy xenografts in our NOD-CB17-SCID mouse mice was also similar to large cell anaplastic medulloblastoma (Fig. 5C). This is consistent with a study reported by Shu et al. (25) where the Daoy cells were injected intracranially into Rag2 Scid mice. Large cell anaplastic histology is often an indication of poor survival in both SHH and Group 3 MB (15). The xenografts were then compared to patient derived MB. Notably their gene signature grouped with SHH subtyped patients (Fig. 5D). The SHH stem cell gene SOX2 (26) was consistently expressed, as was GLI2 another transcription factor involved in SHH signaling.

Discussion

Children diagnosed with MB were more likely to relapse and die if their tumor was classified in either the Group 3 or SHH subtype. This differs somewhat to previously published studies reporting SHH as an intermediate prognosis (5) (7) and we suspect that this could be due to differences in the way patients are treated, such as avoiding radiation in younger patients and/or the composition of the patient cohort. In our study, we only included pediatric MB whereas other reports also included adult tumors (5). Further, the SHH subtype in children and adults are associated with different outcomes (15). One of the limitations of our study was availability of fresh primary tumor tissue from different subtypes. While only SHH and Group 4 tumors were available to culture during this study’s duration, we nonetheless hypothesize that our findings have major clinical implications for many MBs given that PLK1 overexpression is prevalent and cuts-across molecular subtypes. As we continue culturing incoming patient cells we will extend our study to test our findings in more MB.
PLK1 is an oncogenic kinase that confers a growth and survival advantage in cancer cells through its central role in mitosis (27). In multiple cancer models, PLK1 inhibition specifically eliminates the malignant cells while leaving the non-malignant cells unharmed (28-30). Furthermore, PLK1 is highly expressed in cancer cells but not in their normal cell counterparts (31, 32) rendering this kinase a particularly attractive molecular target for cancer therapeutics. Our study tests the PLK1 small molecule inhibitor, BI2536, which has been evaluated in cancer patients (33, 34). Various other small molecule inhibitors to PLK1 have been designed and evaluated in phase I/II clinical trials including BI2536, BI6727, Rigosertib, and GSK461364 (35-39). None of these trials have specifically addressed the possibility that PLK1 inhibitors may be beneficial for the treatment of brain tumors. Our group has demonstrated that PLK1 inhibitors could be used to target glioblastoma (18, 40) and another group has published data agreeing with our findings in MB (41, 42). We illustrate that PLK1 is a promising drug target for MB because 1) it is highly expressed in tumors relative to normal brain tissues and 2) there are small molecule inhibitors that suppress primary MB cells and cell lines in vitro and in vivo.

In conclusion, patients that have tumors expressing very high levels of PLK1 are considered to be at elevated risk for relapse and death. Since there are several PLK1 inhibitors in clinical trials for adult malignancies, we propose that these drugs may also provide benefit for selected MB patients. In the future, it will be desirable to personalize the treatment of MB by selecting patients with high PLK1 using accurate, sensitive methods such as that of the NanoString nCounter technology, where subgroup affiliation of tumors can be assigned rapidly and reproducibly (13). Further, we anticipate that PLK1 inhibitors may have fewer detrimental side effects, as it is not expressed at high levels in normal brain tissue. Therefore, it could be a great improvement to many of the chemotherapies currently being used that can often cause
long-term adverse effects (43). These pre-clinical studies pave the way for improving the treatment of MB through PLK1 inhibition.

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References


Figure Legends

Figure 1. Molecular characteristics of MB dictate outcome and offer potential drug targets  
(A-B) Patients with the SHH subtype of MB had the highest rates of relapse and the worst chance of overall survival, respectively. (C) The patients as well as four MB cell lines were subtyped into the different categories based on gene expression and are represented using a heatmap (Red= high expression, Green= low expression). Cell lines (ONS76, UW228, UW426, DAOY) were all statistically classified as SHH (PAM= 1.0E+00). (*denotes patients from which the indicated primary cell cultures were established). As well, (D) principal component analysis (PCA) uses dimensionality reduction of the 22 minimal marker genes (13) to show association of cultured cells with the MB subgroups. Circles represent individual patients (WNT= orange, SHH=purple, Red=group 3, Purple=group4). Green circles represent cell lines grown as neurospheres (Daoy, ONS76, UW228, and UW426).

Figure 2. PLK1 expression correlates with poor patient survival. (A) A library of 129 small molecule inhibitors was screened against Daoy cells in a 72hr growth assay. (B) NanoString nCounter analysis of 74 patient samples (blue bars) shows PLK1 mRNA is overexpressed in MB compared to normal cerebellum (red line) and indicates cut off between high and low expressers. (C) High PLK1 mRNA expression predicts probability of patient relapse (n=65, P=0.046) and
poor overall survival (n=65, P=0.001), and is (D) validated in a second independent cohort from Toronto, ON (Progression-free survival, n=55, P=0.004; Overall Survival, n=58, P=0.019).

Figure 3. The response of primary MB cells to BI2536 is correlated with the expression of PLK1 in the tumors. (A) Primary cultured MB cells retain gene expression characteristics of the tumor of origin as demonstrated using NanoString analysis of mRNA extracted from primary cultured cells and FFPE tissue from matched origin patient tumors. BTX001 exemplifies a high PLK1 SHH tumor, and BT016 as a low PLK1 Group 4 tumor. (B) PLK1 mRNA from the cerebellum tissue (CB), human neural stem cells (hNSC), patient-derived SHH MB sample BTX001, and patient-derived Group 4 MB BT014. (C) BI2536 (10nM) inhibited the self-renewal of BTX001 (6 day exposure) (scale bar=140μm). BT014 were not responsive to 10nM BI2536 (6 day treatment) (scale bar=140μm). (D) hNSCs were treated with 1, 5 and 10nM of BI2536 for 72hrs, stained with Hoechst dye. BI2536, even at the highest dose tested (10nM), had a negligible impact on their growth.

Figure 4. PLK1 inhibition suppresses cell growth and induces apoptosis in Daoy cells. (A) Daoy cells were treated with 5nM of PLK1 siRNA for 72hrs, knockdown was confirmed by immunoblotting (bottom) and cell growth was assessed by Hoechst staining (top). Immunoblotting for PLK1 substrate, P-TCTP^{Ser46}, following siPLK1 knockdown. Densiometry quantification normalized to Actin housekeeper. (B) P-TCTP^{Ser46}, PARP cleavage, and P-CDC25C^{Ser198} in Daoy cells treated with DMSO, 1, 2.5, 5, or 10nM BI2536 for 48hrs. Daoy cells were treated with 0.5-100nM BI2536 for 24, 48 and 72hrs then growth was assessed by Hoechst staining. (C) Daoy cells were treated with 5nM BI2536 for 24hrs, subjected to flow cytometry.
for analysis of cell cycle profile with propidium iodide. PLK1 was inhibited in Daoy cells using siPLK1 or 10nM BI2536 for 24 hours. The stained for alpha-tubulin (green), pericentrin (red), and DAPI nuclear staining (blue). (D) Daoy cells were treated with 5nM BI2536 for 48 hours. Apoptosis was measured by immunoblotting for PARP cleavage and P-H2AX<sup>Ser139</sup>, or Annexin V-PE/7AAD staining.

**Figure 5. PLK1 inhibition suppresses tumorsphere formation *in vitro* and delayed disease progression *in vivo*.** (A) Daoy cells were treated with vincristine, cisplatin or etoposide for 72 hours and stained with Hoechst for quantification. (B) Daoy cells were transplanted intracranially into mice and treated with BI2536 (n=6) or chemotherapy (n=6) according to a standard-of-care schedule and compared to control (n=6). BI2536-treated mice lived longer than controls (P=0.0142, Log rank) and was comparable to the benefit observed from chemotherapy (P=0.0336). No significant difference in survival was observed between BI2536- and chemotherapy-treated mice (P=0.4205). (C) The Daoy xenografts were characterized as having large cellular morphology, cell wrapping, high mitotic activity, invasion into cerebellum and along the leptomeninges. (D) Heatmap showing Daoy xenograft tumors resembled SHH patient. Gene expression (Green=low expression, Red=high expression) and had large cell anaplastic (LCA) histology.

**Table 1. Univariate and multivariate analyses of clinical, pathological and biological endpoints of the Discovery cohort.**
Figure 1

A. Proportion of Relapse-Free Patients

- **WNT**: Group 4
- **SHH**: Group 3

Log rank P=0.048
Breslow P=0.021

Time in years: 0.0 - 5.0 - 10.0 - 15.0 - 20.0 - 25.0

B. Cumulative Survival

- **WNT**: Group 4
- **SHH**: Group 3

Log rank P=0.010
Breslow P=0.004

Time in years: 0.0 - 5.0 - 10.0 - 15.0 - 20.0 - 25.0

C. Gene Expression Heatmap

- **PAM<sub>SHH</sub> = 1.0**
- **GROUP 4**
- **GROUP 3**
- **SHH**
- **WNT**

D. Cell Viability (%)

- **DMSO**
- **1uM LBH-589 (Panobinostat)**
- **10uM LBH-589 (Panobinostat)**
- **1uM BI 2536**
- **10uM BI 2536**
- **1uM BI 6727 (Volasertib)**
- **10uM BI 6727 (Volasertib)**
- **1uM GSK461364**
- **10uM GSK461364**
- **1uM ARQ-197 (Tivantinib)**
- **10uM ARQ-197 (Tivantinib)**
- **1uM AZD8055**
- **10uM AZD8055**
- **1uM INK128**
- **10uM INK128**

**PLK1 inhibitors**

- **HDAC**
- **AURK**
- **PLK1**
- **c-MET**
- **mTOR**
Figure 2

A. PLK1 Expression in MB compared to Normal Brain

B. PLK1 Expression Predicts Patient Relapse

C. PLK1 Expression Predicts Patient Survival

Log rank $P = 0.004$
Breslow = 0.001

Log rank = 0.003
Breslow $P = 0.003$
Figure 3

A. Relative PLK1 Transcript Level (fold difference) for CB, hNSC, and BTX001.

B. Total Number of Tumourspheres (per well) for DMSO and BI2536, showing significant difference (*).

C. Relative PLK1 Transcript Level (fold difference) for Group 4, showing significant difference (*).

D. Number of Tumourspheres (per well) for BI2536 at 10nM and 20nM, showing significant difference (*).

E. Total Cell Number (per random field counted) for DMSO and BI2536 at 1, 5, and 10nM.

F. Relative PLK1 Transcript Level (fold difference) for various cell lines, showing significant difference (SHH).
Figure 4

A. PLK1-Vinculin

Relative Cell Growth

Control siPLK1

B. Ratio

Ratio

P-TCTP/Actin

P-TCTP

Plk1

Actin

C. BI2536 (nM)

Ratio

P-TCTP/Actin

P-TCTP

PARP

P-CDC25C

Actin

D. Relative Growth

0h 24h 48h 72h (hrs)

E. Percent + P-histone H3 Cells (%)

24hr 48hr 72hr

F. DMSO

%G1=26.7

%S=53

%G2=18.5

BI2536

%G1=7.16

%S=25.6

%G2=53.2

G. Control siPLK1 DMSO BI2536

H. Control siPLK1 DMSO BI2536

P-H2AX

PARP

PARP (cleaved)

Actin

I. 7AAD

Annexin V-PE

Control siPLK1 DMSO BI2536

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Figure 5

A. 

B. Bmi1 Bmi1

C. Relative Cell Growth (fold change)

D. Fraction Survival
Table 1. Summary of the pediatric MB patients included in the study cohort.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>WNT</th>
<th>SHH</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Patients:</td>
<td>74</td>
<td>9 (12%)</td>
<td>21 (29%)</td>
<td>18 (24%)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3 years</td>
<td>20 (27%)</td>
<td>0 (0%)</td>
<td>12 (57%)</td>
<td>7 (39%)</td>
</tr>
<tr>
<td>Between 3 to 8 years</td>
<td>33 (45%)</td>
<td>1 (11%)</td>
<td>8 (38%)</td>
<td>8 (44%)</td>
</tr>
<tr>
<td>Between 8 to 12 years</td>
<td>12 (16%)</td>
<td>4 (44%)</td>
<td>0 (0%)</td>
<td>3 (17%)</td>
</tr>
<tr>
<td>&gt;12-18 years</td>
<td>9 (12%)</td>
<td>4 (44%)</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Average (years)</td>
<td>6.2</td>
<td>11.0</td>
<td>3.7</td>
<td>4.6</td>
</tr>
<tr>
<td>Range (years)</td>
<td>0.3 to 16.8</td>
<td>6.1 to 14.8</td>
<td>0.3 to 16.8</td>
<td>1.6 to 10.2</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>27 (37%)</td>
<td>7 (78%)</td>
<td>9 (43%)</td>
<td>7 (39%)</td>
</tr>
<tr>
<td>Male</td>
<td>46 (63%)</td>
<td>2 (22%)</td>
<td>12 (57%)</td>
<td>11 (61%)</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>39 (53%)</td>
<td>5 (56%)</td>
<td>12 (57%)</td>
<td>8 (44%)</td>
</tr>
<tr>
<td>M1</td>
<td>14 (19%)</td>
<td>1 (11%)</td>
<td>3 (14%)</td>
<td>5 (28%)</td>
</tr>
<tr>
<td>M2</td>
<td>4 (5%)</td>
<td>0 (0%)</td>
<td>2 (10%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>M3</td>
<td>10 (14%)</td>
<td>1 (11%)</td>
<td>1 (5%)</td>
<td>3 (16%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>7 (9%)</td>
<td>2 (22%)</td>
<td>3 (14%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Extent of Resection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gross Total Resection</td>
<td>49 (66%)</td>
<td>6 (67%)</td>
<td>15 (71%)</td>
<td>10 (56%)</td>
</tr>
<tr>
<td>Subtotal or less</td>
<td>22 (30%)</td>
<td>3 (33%)</td>
<td>5 (24%)</td>
<td>6 (33%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (4%)</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy Only</td>
<td>16 (22%)</td>
<td>0 (0%)</td>
<td>9 (43%)</td>
<td>3 (16%)</td>
</tr>
<tr>
<td>Radiation Only</td>
<td>10 (14%)</td>
<td>4 (44%)</td>
<td>3 (14%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Both Chemo and Radiation</td>
<td>44 (59%)</td>
<td>5 (56%)</td>
<td>7 (33%)</td>
<td>13 (72%)</td>
</tr>
<tr>
<td>No Treatment</td>
<td>4 (5%)</td>
<td>0 (0%)</td>
<td>2 (10%)</td>
<td>1 (6%)</td>
</tr>
</tbody>
</table>
Table 2. Univariate and multivariate analyses of clinical, pathological and biological endpoints.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. (n=73)</th>
<th>Log-Rank test (p value)</th>
<th>No. (n=64)</th>
<th>Hazard ratio (95% confidence interval)</th>
<th>Cox regression analysis (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 3 years</td>
<td>20</td>
<td>0.011</td>
<td>15</td>
<td>0.422 (0.122 to 1.463)</td>
<td>0.174 (n.s)</td>
</tr>
<tr>
<td>≥ 3 years</td>
<td>53</td>
<td></td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>46</td>
<td>0.831 (n.s)</td>
<td>40</td>
<td>1.211 (0.450 to 3.255)</td>
<td>0.705 (n.s)</td>
</tr>
<tr>
<td>Female</td>
<td>27</td>
<td></td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Metastasis</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Present</td>
<td>28</td>
<td>0.043</td>
<td>25</td>
<td>3.488 (1.093 to 11.126)</td>
<td>0.035</td>
</tr>
<tr>
<td>Not Present</td>
<td>39</td>
<td></td>
<td>39</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Extent of Resection</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gross Total Resection</td>
<td>49</td>
<td>0.601 (n.s)</td>
<td>45</td>
<td>0.882 (0.317 to 2.455)</td>
<td>0.809 (n.s)</td>
</tr>
<tr>
<td>Subtotal Resection or less</td>
<td>21</td>
<td></td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Radiation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Yes</td>
<td>53</td>
<td>0.021</td>
<td>48</td>
<td>3.519 (0.345 to 35.888)</td>
<td>0.288 (n.s)</td>
</tr>
<tr>
<td>No</td>
<td>18</td>
<td></td>
<td>16</td>
<td></td>
<td></td>
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<tr>
<td><strong>Chemotherapy</strong></td>
<td></td>
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<tr>
<td>Yes</td>
<td>60</td>
<td>0.965 (n.s)</td>
<td>53</td>
<td>3.325 (0.542 to 20.390)</td>
<td>0.194 (n.s)</td>
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<tr>
<td>No</td>
<td>13</td>
<td></td>
<td>11</td>
<td></td>
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<tr>
<td><strong>Combined Treatment</strong></td>
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<tr>
<td>Chemo &amp; Radiation</td>
<td>44</td>
<td>0.429 (n.s)</td>
<td>39</td>
<td>0.661 (0.056 to 7.782)</td>
<td>0.742 (n.s)</td>
</tr>
<tr>
<td>Single or No Treatment</td>
<td>29</td>
<td></td>
<td>25</td>
<td></td>
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<tr>
<td><strong>PLK1 Transcript</strong></td>
<td></td>
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</tr>
<tr>
<td>High</td>
<td>30</td>
<td>0.017</td>
<td>26</td>
<td>3.673 (1.426 to 9.460)</td>
<td>0.007</td>
</tr>
<tr>
<td>Low</td>
<td>43</td>
<td></td>
<td>38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.s = not significant
Personalizing the treatment of pediatric medulloblastoma: Polo-like kinase PLK1 as a molecular target in high-risk children.

Joanna Triscott, Cathy Lee, Colleen Foster, et al.