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TITLE: A Pediatric Brain Tumor Consortium Phase I/ II and Surgical Study of CX-4945 in Patients with Recurrent SHH Medulloblastoma

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SCHEMA

This is a multicenter, Phase I, Phase II, and Surgical study of the CX-4945 drug (silmitasertib sodium) for patients with recurrent SHH (Sonic hedgehog) medulloblastoma.

Brain tumors are the most common solid tumor in the pediatric population and the second leading cause of cancer-related death in children. Of these, medulloblastoma (MB) is one of the most common malignant tumors. Around 20% of MBs are driven by over activity of the Hedgehog (Hh) signaling pathway. Typically, children with SHH medulloblastoma undergo surgical resection followed by craniospinal irradiation and intense multi-agent chemotherapy. While many SHH MBs will respond to current therapies, around 30% will recur, often while still undergoing primary therapy. There are no standard options for tumor recurrence and these patients have a dismal prognosis with <5% long-term survival.

CX-4945 is a tetracyclic synthetically derived small molecule carboxylate acid salt that exhibits potent and highly selective inhibition of Casein Kinase 2 (CK2). The biological activity of CX-4945 has been evaluated in both *in vitro* and *in vivo* studies. Key attributes of CX-4945 include potent inhibition of CK2 enzymatic activity and a highly selective kinase profile. The mode of action of CX-4945 is intracellular inhibition of the CK2 enzyme but no other kinases. Evaluation of CX-4945 in cell viability assays revealed broad spectrum anti-proliferative activity in cancer cell lines and direct suppression of DNA repair pathways.

We have previously shown that treating SHH MBs with CX-4945 results in a dose-dependent loss in tumor cell viability in multiple human MB cell lines. These included cell lines with *TP53* mutations, *MYC* amplification and chromosomal instability, which represent some of the most aggressive forms of MBs. Furthermore, mice harboring flank allografts of vismodegib-resistant MBs had near-complete cessation of tumor growth when treated with CK2 inhibitors. Finally, 43% of mice with cerebellar SHH MB had long-term tumor regression following 30 days of treatment with CX-4945. Therefore, we propose the following studies:

Phase I

The primary objectives of the Phase I part of the study are to find the maximum tolerated dose, recommend a Phase II dose for skeletally-immature children, study the toxicities associated with doses of this drug, define dose limiting toxicities, and characterize the pharmacokinetics (PK) in skeletally-immature children.

Skeletally-immature children with refractory or recurrent medulloblastoma of SHH subgroup will be enrolled on the Phase I component of this trial once it is initiated after a safety cohort of 3 subjects are treated on the skeletally-mature cohort. CX-4945 will be administered twice a day on a continuous basis to skeletally-immature children with refractory or recurrent medulloblastoma of the SHH subgroup. Each course will be 28 days (4 weeks) in duration and the first course will be used as the Dose Limiting Toxicity (DLT) observation period. CX-4945 is available in 200 mg capsules. The starting dose will be Dose Level 1, 600 mg/m² BID which corresponds approximately to the BSA-adjusted adult recommended phase 2 dose (RP2D) of 1000 mg. Dose Level 0 is provided in case the adult RP2D is not well tolerated in skeletally-immature children.

The CX-4945 dose will be increased in an approximately 30% increment to Dose Level 2 in the subsequent cohort if Dose Level 1 is well tolerated. No inpatient dose escalation will be permitted on the protocol. Only DLTs observed during the dose-finding period of therapy will be used to guide dose escalation. Dose escalation will be governed by the statistical design as described in [Section 9](#) of the protocol.

Once the MTD is established, an expansion cohort for pediatric skeletally-immature patients will be opened to better describe the safety, PK, and preliminary efficacy of the maximum tolerated dose (MTD) of CX-4945 in this cohort as described on the [Statistical Section](#).

Phase I Dose Escalation Schedule		
Dose Level	Dose of CX-4945	Eligible BSA Range
Level 0	400 mg/m ² twice a day	≥0.84m ² - ≤2.25m ²
Level 1*	600 mg/m ² twice a day	≥0.60m ² - ≤2.00m ²
Level 2	800 mg/m ² twice a day	≥0.63m ² - ≤2.00m ²
*Starting Dose		

Surgical Study

The primary objective of the surgical part of the study is to characterize the concentrations of CX-4945 in tumor tissue after treatment with CX-4945. The surgical study will be initiated after the first 3 patients in the skeletally-mature cohort are treated for initial assessment of safety. The surgical component will be initiated if no more than 1/3 subjects experience a DLT.

Patients will receive CX-4945 at the established pediatric MTD/adult RP2D for 5-7 days before surgical resection of their tumor, at which time tumor samples will be taken for pharmacokinetic analysis. Skeletally-mature patients with SHH medulloblastoma will be eligible for enrollment as soon as accrual to the surgical study is initiated and will receive the drug at 1000 mg BID or its BSA adjusted equivalent depending on age and BSA criteria as per [Section 6.3](#).

Skeletally-immature children will only be eligible to enroll on the surgical study once the MTD/RP2D is defined in the Phase I part of the study and will receive drug at the established MTD/RP2D dose for this cohort. Following recovery from surgery (no earlier than 2 weeks and no later than 4 weeks), patients will resume CX-4945 for up to 26 cycles if they meet laboratory parameters as defined in [Section 3.2.4.8](#). Patients who meet the criteria for initiation of treatment for the Phase I expansion cohort or Phase II part of the study post-surgery will contribute to the accrual goals of those components. The only exceptions to this rule are the phase II and phase I expansion subjects without measurable disease post-surgery, who will still be treated but will not be counted towards the accrual goals since they will not be assessable for objective response. They will however contribute to all other objectives of the study including the primary toxicity objectives.

Phase II

The primary objectives of the Phase II part of the study are to establish the safety and characterize the toxicity of 1000mg BID or its BSA adjusted equivalent of CX-4945 given continuously in

skeletally-mature patients with recurrent SHH medulloblastoma, and to estimate the objective response rate associated with CX-4945 in skeletally-mature patients with recurrent SHH medulloblastoma.

Skeletally-mature children and adults with SHH medulloblastoma will enroll on the Phase II component of this trial. CX-4945 will be administered twice a day at the adult RP2D, 1000 mg BID or at its BSA adjusted equivalent; however, the dose will be given continuously throughout 28-day courses. The patient will remain on CX-4945 for up to 26 cycles. Accrual to this component will be staggered to ensure that the planned continuous dosing as well as the new formulation is safe in this population. More specifically up to 3 patients will be enrolled initially at Dose Level 1 and will be observed during the first course for any dose limiting toxicities. If no more than 1 patient experiences a DLT then 3 additional patients will be enrolled at Dose Level 1 and if no more than 1/6 patients experience a DLT then accrual will follow the Phase II design. If more than 1/6 patients experience a DLT during the first course, the dose of CX-4945 will be reduced to 800mg BID or at its BSA adjusted equivalent given on a continuous basis.

Phase II Dose Schedule – Skeletally-Mature Patients		
Dose Level	Dose of CX-4945	
	Dosing for patients ≤ 18 years and BSA $\leq 1.74\text{m}^2$	Dosing for patients > 18 years or BSA $> 1.74\text{m}^2$
Level 0	400 mg/m ² twice a day	800 mg twice a day
	Dosing for patients ≤ 18 years and BSA $\leq 1.50\text{m}^2$	Dosing for patients > 18 years or BSA $> 1.50\text{m}^2$
Level 1*	600 mg/m ² twice a day	1000 mg twice a day
* Starting Dose		

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1.0 OBJECTIVES

1.1 Primary Objectives: Phase I

1.1.1 To estimate the maximum tolerated dose (MTD) and recommend a Phase II dose of CX-4945 administered orally daily to skeletally-immature children with recurrent SHH (sonic hedgehog) medulloblastoma

1.1.2 To describe the toxicity profile and define the dose-limiting toxicities associated with CX-4945 in children with recurrent SHH (Sonic hedgehog) medulloblastoma

1.1.3 To characterize the pharmacokinetics of CX-4945 administered on this schedule in skeletally-immature children with recurrent SHH medulloblastoma

1.2 Primary Objective: Surgical Study

1.2.1 To characterize the concentrations of CX-4945 in tumor after administration of CX-4945 and surgical resection

1.3 Primary Objectives: Phase II

1.3.1 To establish the safety and characterize the toxicity of 1000mg BID continuous dosing of CX-4945 in skeletally-mature patients with recurrent SHH medulloblastoma

1.3.2 To estimate the objective response rate associated with CX-4945 in skeletally-mature patients with recurrent SHH medulloblastoma

1.4 Secondary Objectives: Phase I

1.4.1 To document preliminary antitumor activity of CX-4945 in skeletally-immature children with recurrent SHH medulloblastoma

1.4.2 To perform a genomic analysis within the confines of a Phase I study to investigate correlation between response to treatment and the presence of specific genomic alterations, and/or specific subgroups of disease

1.5 Secondary Objective: Surgical Study

1.5.1 To explore the ability of CX-4945 at the MTD/ RP2D to inhibit CK2-mediated signaling in tumor.

1.6 Secondary Objective: Phase II

1.6.1 To characterize the pharmacokinetics of CX-4945 in skeletally-mature patients with recurrent SHH medulloblastoma

1.6.2 To perform a genomic analysis within the confines of a Phase II study to investigate correlation between response to treatment and the presence of specific genomic alterations and/or specific subgroups of disease

2.0 BACKGROUND

2.1 Study Disease(s)

Brain tumors are the most common solid tumor in the pediatric population and the second leading cause of cancer-related death in children.¹ Of these, medulloblastoma (MB) is the most common malignant tumor accounting for around 20% of all childhood brain tumors.² Around one third of MBs are driven by over activity of the Hedgehog (Hh) signaling pathway.³ Typically, children with SHH medulloblastoma undergo surgical resection followed by craniospinal irradiation and intense multi-agent chemotherapy. While many SHH MBs will respond to current therapies, around 30% will recur, often while still undergoing primary therapy.⁴ There are no standard options for tumor recurrence and these patients have a dismal prognosis with <5% long-term survival.⁴

2.2 CX-4945

CX-4945 is a tetracyclic synthetically derived small molecule carboxylate acid salt that exhibits potent and highly selective inhibition of CK2. The biological activity of CX-4945 has been evaluated in both *in vitro* and *in vivo* studies. Key attributes of CX-4945 include potent inhibition of CK2 enzymatic activity and a highly selective kinase profile. The mode of action of CX-4945 is intracellular inhibition of the CK2 enzyme but no other kinases. Evaluation of CX-4945 in cell viability assays revealed broad spectrum anti-proliferative activity in cancer cell lines and direct suppression of DNA repair pathways.⁵

2.2.1 Preclinical Studies

2.2.1.1 Anti-tumor activity

A. Casein Kinase (CK2) and Oncogenesis

Protein kinase CK2 is a constitutively active serine/threonine kinase that has been much studied as a pro-survival, antiapoptotic kinase. CK2 plays a multifunctional role in cell cycle regulation, signal transduction, transcriptional control, angiogenesis, apoptosis and inflammation, cellular processes that have been implicated in the establishment and maintenance of the cancer phenotype. CK2 catalyzes the phosphorylation of a large number of proteins, thus modulating their activities. CK2 is ubiquitously distributed in the cytoplasmic and nuclear compartments of multiple cell types.⁶⁻⁸ Owing to its internal molecular structure the enzyme is constitutively active and does not need phosphorylation by other kinases for activation. The elevation of CK2 activity is often due to overexpression of the molecule. Unlike other signaling molecules such as PI3K, PTEN, RAF and RAS, where genetic alterations lead to an altered molecule concomitant with a dysregulation of their pathways, no CK2 mutations have been found to date. While no mutations in CK2 have been reported, CK2 is known to be amplified in glioblastoma and its activity and expression levels are elevated in many cancers of diverse genetic background including breast, lung, prostate, colorectal and leukemias. Moreover, the elevation of CK2 in cancer tissues has frequently been linked to disease progression and poor prognosis.^{7,9} Given the wide spread overexpression of CK2 in multiple cancers and its role in multiple non-oncogenic processes required to sustain the cancer phenotype, a selective inhibitor of CK2 is an attractive targeted approach to treating cancer.

B. The Role of CK2 in the Hedgehog Signaling Pathway and in MB

A consensus has been reached to recognize at least 4 subgroups of MB with distinct gene expression profiles and clinical features which includes prognosis and demographics. The WNT (wingless) group of MB accounts for around 10% of tumors.¹⁰ These tumors occur in older children and adolescents with a peak incidence of around 10-12 years and carry an excellent prognosis with survival rates ranging 90-95%.^{11,12} Sonic hedgehog (SHH) and MYC-amplified medulloblastomas (Group 3/4) make up the majority of treatment-resistant medulloblastomas.^{10,13} Twenty five to thirty percent of all MB are driven by the Hedgehog (Hh) signaling pathway and the majority of Hh-driven MB occurs in children <3 years of age.¹⁴ This represents a particularly vulnerable patient population who is especially prone to the neurocognitive deficits that result from treatment. Given the unique challenges of management in this population, MB in very young children has been described as one of the ‘orphan diseases’ that will most benefit from targeted therapy that can reduce the current toxicities associated with traditional non-targeted approaches.

Given its role in MB, drugs targeting the Hh pathway have been developed, largely focused on inhibiting the seven-pass transmembrane protein smoothened (SMO). Vismodegib, a first-in-class SMO inhibitor, has been shown to be well tolerated in phase I pediatric trials and dramatic initial responses were observed in some patients in phase II trials.⁴ While this demonstrated that Hh inhibition is sufficient to drive tumor regression, unfortunately all responses were transient and tumors recurred at around 3 months. This was found to be largely a result of a point mutation in the vismodegib target, SMO, which resulted in a 100-fold decrease in drug binding, thereby allowing Hh pathway re-activation.¹⁵ Furthermore, large-scale genome sequencing of medulloblastoma has revealed that nearly half of Hh-driven MB have activation of the Hh pathway downstream of SMO and are predicted to be non-responsive to SMO inhibitors such as vismodegib.¹³ Thus, there is tremendous unmet clinical need to develop Hh pathway inhibitors that target signaling downstream of SMO.

Using a mass spectrometry-based phosphoproteomic approach, we have discovered a novel and critical role for the protein kinase CK2 in MB growth and survival. We found that CK2 was responsible for hundreds of phosphorylation events occurring in medulloblastoma, the most important of these being its role as a regulator of the Hh signaling pathway. In Hh signaling, Hh ligands (SHH, Ihh, Dhh) bind and inhibit the cell surface receptor complex including Patched (Ptch1).^{16,17} This results in activation of the G protein-coupled receptor Smo^{18,19} and consequently activation of the otherwise latent Gli2 zinc-finger transcription factor^{20,21} via suppression of suppressor of fused (Sufu4)²² (Figure 1a). Gli2 drives GNP proliferation by controlling transcription of target genes such as *Gli1* and the cell cycle regulators *Ccnd1* and *Mycn*. Genetic and pharmacological studies determined that CK2 is required for Hh signaling (Figure 1b, c). Epistasis studies, as described in Figure 1, demonstrate that CK2 affects the terminal-most Hh signaling components by promoting Gli2 stability and Gli2’s interaction with target genes (Figure 1d-f). This circumvents challenges of emergence of resistance and a priori resistance seen with existing small molecule inhibitors targeting upstream at SMO.

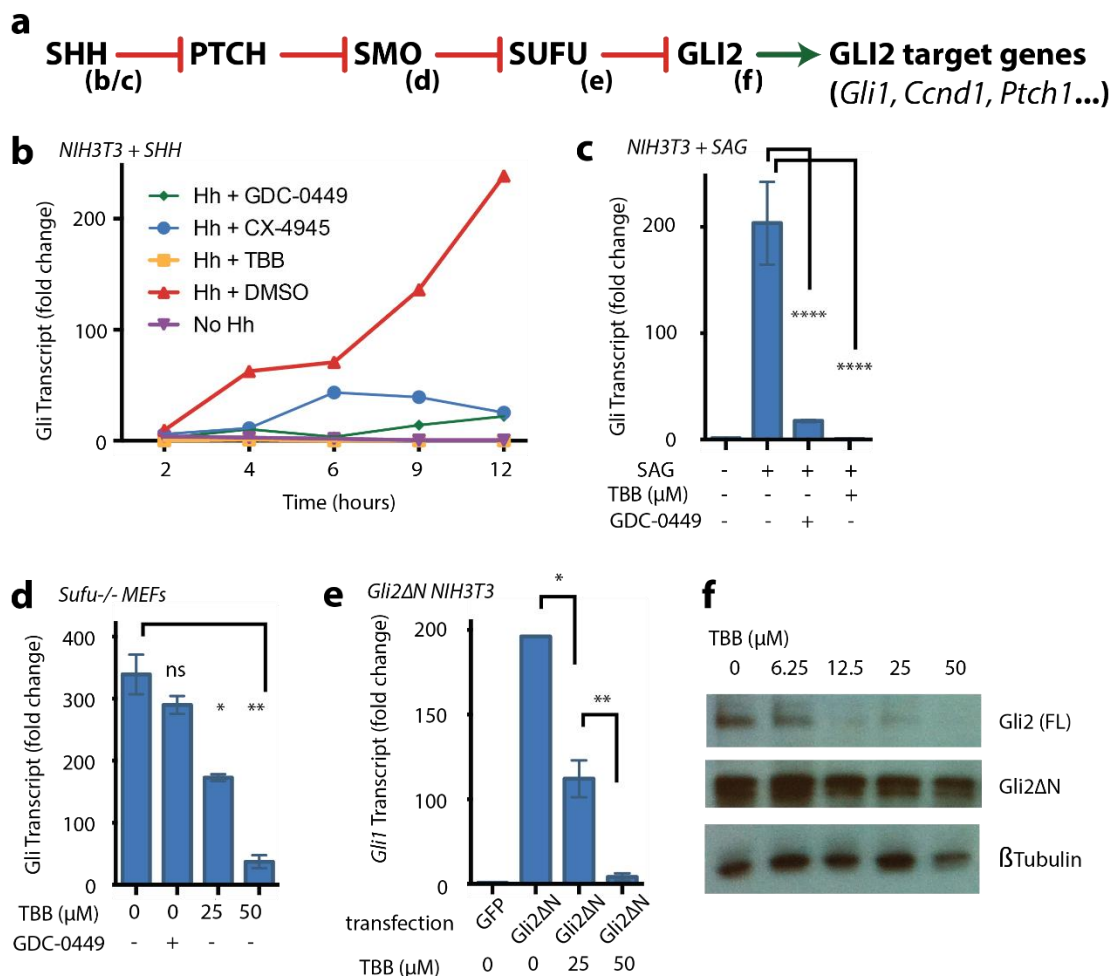


Figure 1: The Role of CK2 in Hedgehog Signaling

(a) Schematic of the canonical hedgehog signaling pathway. Letters represent targets of epistasis studies performed in Fig 1b-g.

(b) SHH protein treatment of NIH 3T3 cells results in the expected increase in *Gli1* mRNA levels, increasing over 12h of exposure. Treatment with CK2 inhibitors, TBB²³ and CX-4945²⁴ or SMO inhibitor GDC-0449 results in a significant decrease in Gli1 transcript levels as early as 4h post exposure.

(c) NIH 3T3s were stimulated with SAG, a Smo agonist.²⁵ The resultant increase in Gli1 transcript was inhibited by both TBB and GDC-0449.

(d) *Sufu*^{-/-} mouse embryonic fibroblasts (MEF)²⁶, which bypass the requirement for Smo activation by SHH, were treated with both TBB and GDC-0499. While GDC-0449 treatment resulted in no significant decrease in Gli1 transcript as expected, TBB treatment still resulted in a dramatic decrease in Gli1 transcript.

(e/f) NIH3T3's were transfected with a murine form of Gli2 that lacks the N terminus (HA-Gli2ΔN)²⁷This results in stable expression of an activated and constitutively nuclear form of Gli2 that lacks sites required for ubiquitin-driven degradation. The resultant increase in *Gli1* mRNA levels was still inhibited by TBB (f). Notably, we did not observe any decrease in Gli2ΔN protein after TBB treatment however there was a decrease in endogenous, full length *Gli2* mRNA levels. This difference in effect on protein stability in full-length vs. Gli2ΔN suggests that CK2 inhibition affects endogenous full-length Gli2 stability as previously described²⁸, however it also has a secondary effect downstream of Gli2 stability. These results suggest CK2 is modulating Hh signaling at the level of Gli2 interaction with target genes and / or target gene transcription.

Legend: ns = p>0.05, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001

2.2.1.2 *Animal Toxicity*

C. Nonclinical Toxicology Studies

The CX-4945 non-clinical safety program included good laboratory practice (GLP), non-GLP, and single and repeated dose toxicity studies in rats and dogs. The acute and sub-acute dose-ranging studies conducted in rats and dogs provided the rationale for the doses to be selected in the GLP 21-day repeated dose toxicology study of CX-4945 (with 7-day recovery groups) administered twice a day in these species.

No bone marrow, cardiac, pulmonary or central nervous system toxicities were observed in any of the CX-4945 non-clinical studies. CX-4945-induced effects and changes were evident in both species tested in the GLP 21-day repeated dose toxicology studies. At the highest doses, lower mean body weights and lower weight gains were observed (with decreased food consumption in dogs), but these effects were resolved following the recovery period. Transient, minimal laboratory changes were noted in rats and dogs at the highest dose level, suggesting hepatic and renal effects, but these resolved following the recovery period. Histopathologic changes were seen at the higher dose levels in salivary glands and bile ducts in rats, and kidney, pancreas, spleen and thymus in dogs; all these changes resolved during the recovery period, except thymic atrophy that remained evident in one of two recovery male dogs.

The beagle dog appears to be the most sensitive species showing more drug related changes, and in one instance (e.g., thymic atrophy) the change persisted beyond the 7-day recovery period. Calculation of the human starting dose for the phase I clinical trial concluded that patients should begin by receiving 90 mg per dose, administered twice a day, in cycles of 21 consecutive days of oral dosing followed by seven days of rest.²⁹

D. Acute and Subacute Tolerability Studies

Early exploratory tests using GLP drug substance were conducted with oral administration of CX-4945 to rat models, as follows:

- A non-GLP dose range (400, 700 and 1,000 mg/kg) finding study in male and female rats to determine the single oral maximum tolerated dose (MTD).
- A non-GLP safety and tolerance study of five consecutive, twice daily oral administrations at several defined dose levels (100, 250, 375 and 750 mg/kg) below the single dose MTD.

Single CX-4945 oral doses up to 1,000 mg/kg (equivalent to 6,000 mg/m²) were well tolerated in dose ranging studies with the drug substance. Subsequently, in the study of five consecutive, twice daily oral doses at 750 mg/kg (equivalent to 4,500 mg/m²) all male rats died (two on day 2, two on day 3 and one on day 4) and one female rat was also found dead (on day 2). Two additional female rats (one dosed at 750 mg/kg, and the second at 375 mg/kg) successfully completed five consecutive days of twice daily oral doses but died very shortly after blood sample collection for chemical pathology. Test drug associated observations (e.g., decreased activity, hunched posture, stained haircoat) were more common in male than in female rats dosed orally with 750 and 375 mg/kg twice daily. The hematology and coagulation studies in treated animals were largely unremarkable when compared with vehicle control animals, except that reticulocyte counts in males and females at the 375 mg/kg level appeared lower. Blood chemistry results showed that treated animals had higher liver transaminase and bilirubin levels when compared with vehicle

control animals. There were no significant necropsy observations associated with CX-4945 administration. The study of five consecutive, twice daily oral doses was repeated with lower doses of 250 mg/kg (equivalent to 1,500 mg/m²) and 100 mg/kg (equivalent to 600 mg/m²). At these lower doses, minimal changes were observed in the monitored parameters. Males at the 250 mg/kg dose lost body weight, and all CX-4945 treated animal groups had decreased food consumption. Mean reticulocyte counts were lower, and mean phosphorus was higher in animals treated at the 250 mg/kg dose level. No other significant observations were evident with these lower doses.

At the conclusion of these acute and subacute tolerability studies in rats, the CX-4945 doses selected for the GLP 21-day repeated dose toxicology study of CX-4945 administered twice a day by oral gavage in Sprague-Dawley rats with a 7-day recovery period were: 0, 35, 100 and 300 mg/kg per dose twice a day (for a total daily CX-4945 dose respectively of: 0, 70, 200 and 600 mg/kg).²⁹

2.2.1.3 Preclinical Pharmacology

E. Preclinical studies of CK2 inhibitors in SHH MB

To examine the clinical significance of CK2 activity in Hh-driven MB, we investigated the effect of CK2 inhibitors on the growth of MB cells *in vitro* and *in vivo*. MB cells derived from *Ptch*^{+/-} mice are dependent on on-going Hh signaling for survival.¹⁷ Three *Ptch*^{+/-} cell lines derived from distinct mouse models demonstrated a dose-dependent loss in viability after 5 days of treatment with either TBB²³ and CX-4945²⁴ two structurally distinct but highly selective CK2 inhibitors (Figure 2a,b), both of which inhibit ATP binding in the catalytic cleft of *csnk2a1* (Figure 2c). In contrast, cell lines derived from diffuse intrinsic pontine glioma (DIPG) another aggressive pediatric brain tumor, are not Hh-dependent and showed no cell death in response to CX-4945 (Figure 2b).

We next investigated the effect of CK2 inhibition in mice harboring MB allografts (Figure 2d-g). TBB administration (30 mg/kg IP BID) resulted in significant inhibition of MB flank allograft growth, as early as 3 days after initiating treatment (Figure 2e). To test efficacy of CK2 inhibitors against SMO inhibitor-resistant MBs, we administered TBB (30 mg/kg IP BID) to mice harboring allografts of MB cells derived from *Ptch*^{+/-}; *Tpr53*^{-/-}; *SmoD477G* murine tumors, which contain a point mutated Smo (*SmoD477G*) that renders them resistant to vismodegib (GDC-0449). Mice harboring flank allografts of these MB cells showed near-complete cessation of tumor growth in response to TBB (Figure 2f,g). Two treatment and control treated mice were sent for histopathological assessment and no treatment-related morbidity was identified. Finally, mice with *Ptch*^{+/-}; *Tpr53*^{-/-}; *SmoD477G* cerebellar tumors were randomized and treated with CX-4945 (37.5 mg/kg PO BID) or vehicle control (DMSO IP BID) by a blinded collaborator. While 100% of control mice died by day 17, 43% of mice treated with CX-4945 administration had long-term regression following 30 days of treatment (Figure 2h). The cerebellum of one treated mouse was sent for histopathology at day 100 and no residual tumor was identified. The remaining two treated mice continued to survive beyond day 250.

The major limitation of previous hedgehog inhibitors has been both acquired and a priori resistance due to mutations occurring downstream of the drug target SMO or at the level of SMO itself. Given the multiple roles of CK2 on both Gli2 stability and Gli2 target gene transcription, the terminal step of the hedgehog signaling pathway, CK2 inhibition is unlikely to be overcome by downstream

mutations within the pathway. However, resistance could still occur due to mutations at the level of CK2 itself. To investigate potential for evolved resistance, three *Ptch*^{+/-} MB cell lines were passaged in TBB, CX-4945 or control (DMSO) for one month. While all cell lines maintained sensitivity to CX-4945, one cell line, MB55, evolved resistance to TBB while maintaining sensitivity to CX-4945. Sequencing of the TBB-resistant MB55 cell line revealed four missense mutations not present in the other cell lines, including the parental TBB-sensitive MB55 cell line: D175:N175, R47:M47, H183:R183, G199:P199, S217N:217). Because the crystal structure of *csnk2a1* is known³⁰ including binding studies with both CX-4945²⁴ and TBB³¹ molecular dynamics simulations could be performed to investigate the role of the different mutations on TBB and CX-4945 binding. It was discovered that the D175:N175 mutation was sufficient to explain an increase of the ATP cavity volume by about 137 Å. The effect of this increased volume and change in catalytic architecture did not affect the binding of ATP or CX-4945 but is detrimental to the retention of TBB. These findings, in combination with the long-term regression observed in mice with cerebellar MB allografts treated with CX-4945, favor the use of CX-4945 over TBB in a clinical setting.

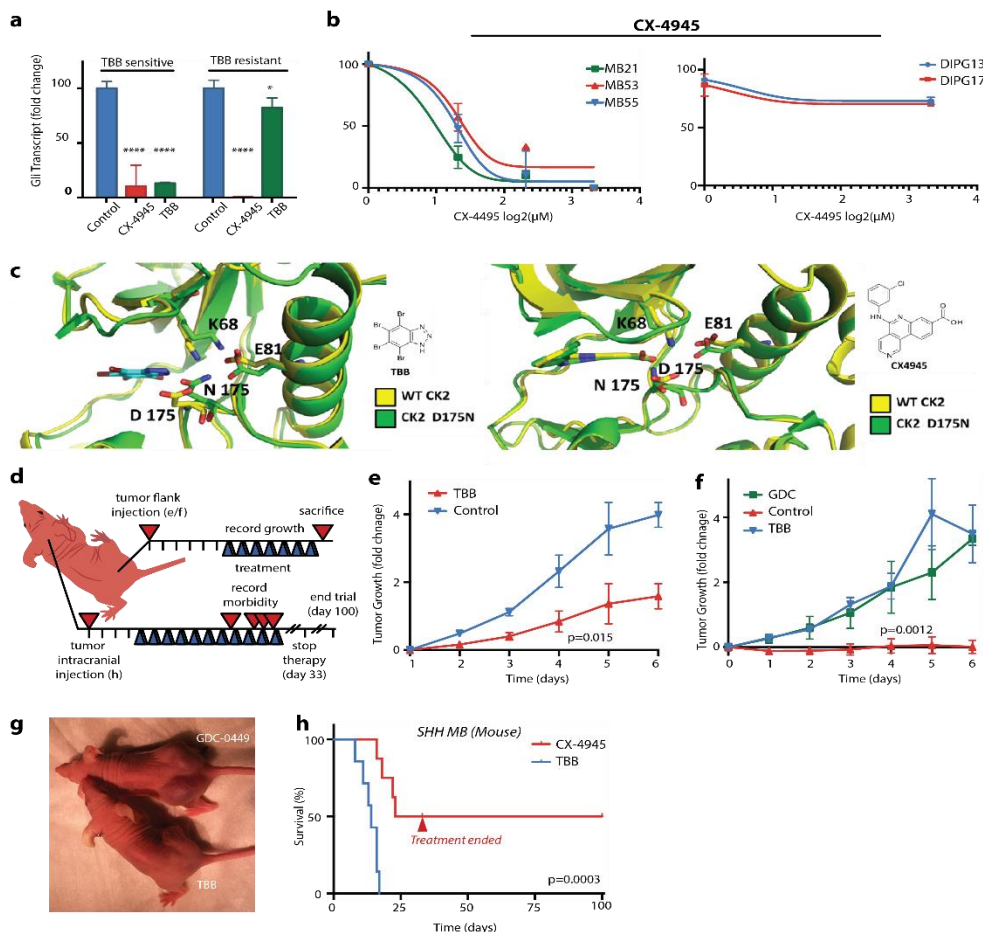


Figure 2: The CK2 Inhibitors in MB

(a/b) CK2 inhibitors TBB and CX-4945 decrease Gli1 transcript levels and have a dose-dependent effect on cell viability in multiple MB cell lines derived from primary MB generated in *Ptch*^{-/-} and *Ptch*^{+/-}; *Trp53*^{-/-} mice. TBB resistance occurred in one *Ptch*^{-/-} cell line MB55, though cells remained sensitive to CX-4945.

- (c) Molecular dynamics simulations demonstrate that the D175:N175 mutation found uniquely in the TBB resistant MB cell line resulted in an increase of the ATP cavity volume by about 137 Å. The effect of this increased volume and change in catalytic architecture did not affect the binding of CX-4945 but is detrimental to the retention of TBB.
- (d) *In vivo* experimental design for tumor flank and cerebellar tumor allografts. In both cases, tumors were allowed to engraft for 3 days (cerebellar studies) or 7 days (flank studies) prior to the initiation of treatment. Flank tumors were measured daily and growth was recorded until control mice met euthanasia criteria at which time all mice were sacrificed (red triangle). Mice with cerebellar tumors were followed daily for morbidity and date of mortality was recorded (red triangles). In both studies, animals were treated twice daily with TBB, CX-4945, or, control (blue triangles) for up to 33 days.
- (e) Mice harboring *Ptch*^{+/-};*Trp53*^{-/-} MB flank allografts treated with TBB have decreased tumor growth relative to vehicle control.
- (f) Mice harboring flank MB allografts derived from MB generated in *Ptch*^{+/-};*Trp53*^{-/-};*SmoD477G* were treated with TBB, GDC-0449, or vehicle control. TBB treated mice showed significant response over control and GDC-0449 treated mice.
- (g) Representative picture of mice treated with TBB (bottom) vs GDC-0449 (top).
- (h) Kaplan-Meier survival analysis of mice harboring intracranial allografts of *Ptch*^{+/-};*Trp53*^{-/-};*SmoD477G* MBs treated with CX-4945 and vehicle control.
- Legend: ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

F. CX-4945 Preclinical Pharmacokinetic Studies

Although thorough formal absorption studies remain to be completed, Son and colleagues used an *in vitro* MDCK assay for the prediction of intestinal absorption of CX-4945. They found that CX-4945 exhibited a high apparent permeability coefficient (P_{app}) value (10.7 ± 0.99 cm/s) at 50 μ M when metoprolol and atenolol exhibited 12.9 ± 0.07 and 0.07 ± 0.01 cm/s, respectively.³² They also reported the extent of absolute bioavailability of CX-4945 in Sprague-Dawley rats after an intravenous and oral dosage of 10 mg/kg was 79.1%.³² The results of *in vivo* oral bioavailability studies in mice (20%) and dogs (40%) further suggest adequate oral absorption.²⁹

Studies of CX-4945 distribution have focused on its binding to cellular blood compartments and plasma with little data available to describe its binding to tissues. CX-4945 is highly bound (98 to 99%) to rat and human plasma proteins, and this binding is independent of CX-4945 concentration. CX-4945 partitions equally into the cellular blood compartment and plasma. Whole-body autoradiography data suggest CX-4945 may not have significant blood-brain barrier penetration. *In vivo* pharmacokinetic studies have shown a steady-state volume of distribution ranging from 1.4 to 18 L/kg, suggestive of moderate to high tissue distribution, but without any report of specific tissues to which it binds.^{29,32}

Limited *in vitro* drug metabolism studies have been conducted using rat and human hepatocytes. The primary metabolic pathway was identified as glucuronidation, however, the actual metabolites have not been identified or quantitated. Qualitatively, the results suggest that conjugation is the primary metabolic pathway (see **Table 1**). In pre-clinical species, CX-4945 exhibited low to moderate clearance ($CL_s = 0.1$ -2.5 L/hr/kg). The mouse showed the highest clearance while rat and dog values were indicative of low to moderate clearance.²⁹

Table 1: Metabolites Identified Upon Treatment of Human Hepatocytes with CX-4945

Metabolite Name	Area %
Parent	8.28

Glucuronide conjugation	60.97
Hydroxylation + glucuronide conjugation	21.57
Hydroxylation + sulfation	1.41

The *in vitro* potential for CX-4945 to inhibit CYP450-mediated metabolism of canonical substrates was evaluated after incubation of CX-4945 with isolated CYP450 isoenzymes (CYPs 1A2, 2C9, 2C19, 2D6, and 3A4). No detectable inhibition was observed for CYP2C19 and CYP2D6. Moderate inhibition was observed for CYP1A2 with an IC₅₀ value > 10 µM. Weak inhibition was observed for CYP2C9 and CYP3A4-testosterone as the IC₅₀ values were > 50 µM. CYP3A4-midazolam exhibited positive cooperativity for this assay. Overall, these results suggest minimal risk for CX-4945 to inhibit drugs metabolized by these pathways in patients and can therefore be safely co-administered with drugs that are metabolized via this pathway. However, no studies were conducted to evaluate the *in vitro* potential for CX-4945 to induce CYP450-mediated drug metabolism.²⁹

In vivo pharmacokinetic studies have been conducted in mice, rats, and dogs and the disposition parameters reported, however, the specific elimination pathways for CX-4945 have not been determined. The terminal half-life in rats ranges from 11.6 hr after intravenous administration to 12.3 hr after oral administration. In dogs, the terminal half-life ranges from 8.3 hrs after intravenous administration to 5.2 to 9.9 hr after oral administration.²⁹ In mice, the area under the plasma concentration-time curve (AUC) for CX-4945 dosed at 76.4 mg/kg was 8181 ng/ml*hr. Assuming linearity, the dosage used in the *in vivo* experiments that led to tumor regression in flank and cerebellar allografts (37.5 mg/kg, [Section 2.2.1.3](#)) is estimated to lead to a plasma systemic exposure of ~4040 ng/ml*hr. (For comparison the MTD in the human phase I trial had a median plasma systemic exposure of 4710 ng/ml*hr).²⁹

2.2.2 Adult Clinical Trials

2.2.2.1 Phase I Studies

G. Phase I Studies of CX-4945 Monotherapy in Solid Tumors

CX-4945 was evaluated in 51 adult patients with advanced solid tumors excluding patients with CNS disease. Patients were administered CX-4945 following a twice-daily (BID) or four-times-daily (QID) dosing regimen for 21 days followed by 7 days of rest. This study demonstrated CX-4945 to be safe and tolerable in patients up to 1000 mg BID and 600 mg QID. The predominant toxicities were gastrointestinal disorders. The primary dose limiting toxicities (DLTs) were diarrhea and hypokalemia. Most toxicities related to CX-4945 were manageable with drug discontinuation, use of antidiarrheal medication and potassium supplementation.

Overall, 35 out of 51 patients (68.6%) reported drug-related adverse events (AE). The most frequently reported drug-related AEs (≥10%) included diarrhea (58.8%), nausea (17.6%), hypokalemia (17.6%), vomiting (15.7%) and fatigue (11.8%). Eleven patients (21.6%) reported drug-related severe AEs: 6 patients (11.8%) reported grade 3 or greater diarrhea, 4 patients (7.8%) reported grade 3 or greater hypokalemia, and 1 patient reported grade 3 or greater lymphopenia.

Although no complete or partial response was reported, stable disease was achieved in 17.3% of the patients. Progressive disease was noted in 53.8% of the patients, and 28.8% of the patients

were not evaluable due to symptomatic progression and lack of tumor assessments. No deaths related to CX-4945 were reported.²⁹

H. Phase I/ II Studies of CX-4945 Monotherapy in Multiple Myeloma

Eleven patients with multiple myeloma were administered CX-4945 orally BID or QID administered for 21 days followed by a 7-day rest period in 28-day cycles. One patient had a DLT, grade 3 diarrhea, at 1000 mg BID. Otherwise, the 3 patients who were treated at 1000 mg BID experienced mostly drug-related mild diarrhea and nausea. The sponsor chose to stop patient enrollment at 1000 mg BID when it was determined that the safety and tolerance profile of CX-4945 was substantially similar to that observed in the solid tumor phase I study, and that dose escalation beyond 1000 mg BID would not be feasible. No official MTD was determined, no further dose escalation occurred and 1000 mg BID administered for 21 days followed by a 7-day rest period in 28-day cycles was declared the recommended phase II dose (RP2D). Eight patients with multiple myeloma had stable disease, no patients had a complete or partial response.²⁹

I. Phase IB Study in Cholangiocarcinoma

A multi-center Phase IB study of gemcitabine + cisplatin and CX-4945 was conducted in 19 patients with advanced cholangiocarcinoma patients. MTD and RP2D of this combination was found to be gemcitabine (1000 mg/m²) and cisplatin (25 mg/m²), both on days 1 and 8 every 21 days with CX-4945 given twice daily on days 0,1,2 and 7,8,9 at 1000 mg. No dose limiting toxicities were encountered. No grade 3 or 4 toxicities at a prevalence > 10% were encountered, and the overall toxicity profile of the combination was reflective of standard gemcitabine and cisplatin toxicity profile. At data cut-off, preliminary efficacy evaluation demonstrated disease control rate of 64% (partial response: 32%, stable disease: 32% and complete response: 0%), along with median progression-free survival (PFS) of 5 months (range 0-14 months).³³

J. Basal Cell Carcinoma (Protocol CX-4945-07)

The on-going phase I clinical trial is a multi-center, open-label, treatment duration increment, expansion, safety, and pharmacodynamic study of CX-4945 administered orally twice daily to patients with advanced basal cell carcinoma (BCC). The primary objective of this study is to determine the RP2D and schedule of CX-4945 when administered orally twice daily for 28 consecutive days, in a 4-week (28 days) cycle, in patients with locally advanced or metastatic BCC. After identification of the RP2D and schedule for CX-4945 in this patient population, ten (10) additional patients with locally advanced BCC and ten (10) patients with metastatic BCC will be treated to assess preliminary evidence of antitumor effects.

2.2.3 Pediatric Clinical Trials

No pediatric studies with CX-4945 have been conducted.

2.3 Rationale for proposed pediatric study

Pediatric patients with recurrent SHH MB have a poor prognosis, and there is a clear need to develop more effective therapies. In view of the preclinical activity of CK2 inhibitors including CX-4945 in SHH MB and the important role played by CK2 in this pathway, we propose the following studies: Phase I study of CX-4945 in skeletally-immature patients with recurrent SHH MB; a Phase II study of CX-4945 in skeletally-mature patients with recurrent SHH MB; Surgical study of CX-4945 in patients undergoing surgical resection. To achieve continuous inhibition of

the SHH pathway, CX-4945 will be administered daily continuously in the Phase I and Phase II studies.

Hypothesis:

Phase I: CX-4945 will have a similar toxicity profile in skeletally-immature children with SHH medulloblastoma to the toxicity profile observed in adults. In this patient population, the MTD or recommended Phase II dose of CX-4945 will be comparable to the adult MTD. Phase II: CX-4945 will be safe when administered at 1000 mg BID continuously and will demonstrate anti-tumor activity in skeletally-mature patients with recurrent SHH medulloblastoma.

Skeletal Toxicity Monitoring in Skeletally-Immature Children:

The hedgehog pathway is known to play a critical role in skeletal development and even brief exposure to Hh inhibitors in juvenile mice has been shown to cause permanent defects in bone growth.³⁴ The permanent defects in bone growth observed in preclinical studies of Hh inhibitors were not observed in early phase clinical studies of vismodegib in children. However, there has since been a report of 3 patients demonstrating widespread and persistent growth plate fusion following prolonged exposure to vismodegib resulting in profound short stature and disproportionate growth.³⁵ Similar bone-related toxicities were reported in association with another SMO inhibitor, sonidegib, in 3 additional pediatric patients.³⁶ Given the concerns regarding bone toxicities with SHH pathway inhibition, baseline and serial knee MRIs will be performed in all skeletally-immature patients on this trial to monitor for bony abnormalities. Bone age X-rays will be obtained if growth plate abnormalities are observed on MRI and will be repeated as clinically indicated.

2.4 Correlative Studies Background

2.4.1 Rationale for Biology Studies

2.4.1.1 Hypothesis

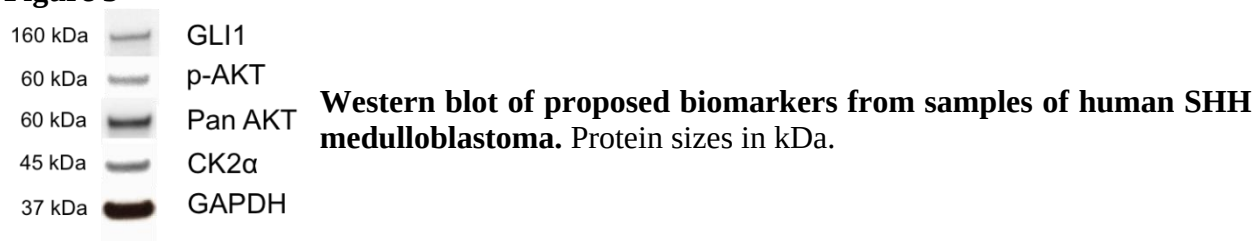
As described in the preclinical studies, we have demonstrated that CK2 is required for hedgehog pathway activation which is, in turn, required for SHH medulloblastomas growth and survival (Figure 1). We go on to demonstrate that CK2 inhibition is sufficient to cause long-term regression of SHH MBs (Figure 2). Biomarker studies will be used to assess whether CX-4945 effectively inhibits CK2 activity and thereby hedgehog activity in tumor tissue of patients with SHH MBs. CK2 activity will be assessed in SHH MB tumor biopsies of patients treated with CX-4945 by measuring levels of phosphorylation of AKT (S129, S473), which is a well-established CK2 substrate.³⁷ This biomarker was similarly used in Phase I studies of CX-4945 in solid tumors. We will also assess the effect of CK2 inhibition on hedgehog pathway inhibition by measuring total protein levels of the well-established terminal Hh pathway target Gli1. Gli1 is a commonly used as a biomarker of Hedgehog pathway activity in SHH MB.^{38,39}

Taken together, these assays will provide insight into drug mechanism and efficacy, and may provide a predictive indices of patient response.

2.4.1.2 Preclinical and Clinical Data

Preclinical *in vitro* and *in vivo* assays have demonstrated that antibodies can reliably recognize the human epitopes of pAKT (S129, S473), AKT and GLI1 in human MB cell lysate (**Figure 3**).

Figure 3



Furthermore, treatment of SHH MB cells with CX-4945 demonstrates a robust and reproducible dose and time-dependent decrease in pAKT and Gli1 that recovers 48h following treatment (Figure 4, Figure 5).

Figure 4

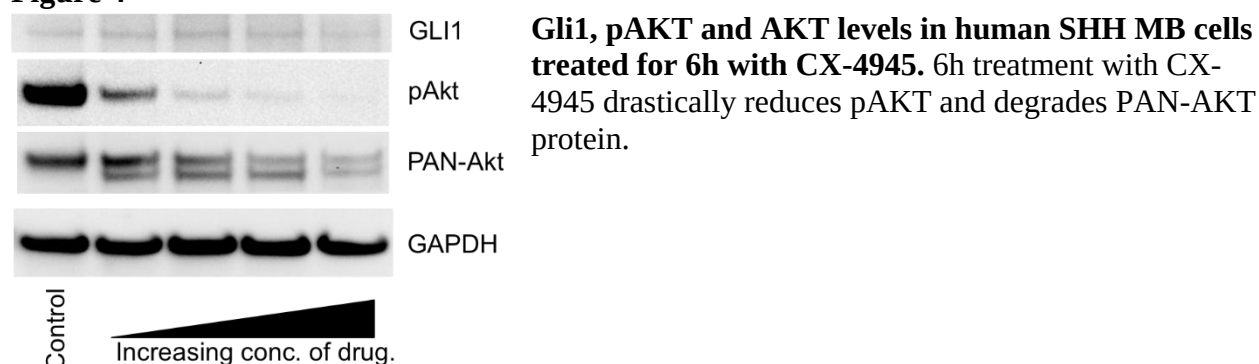
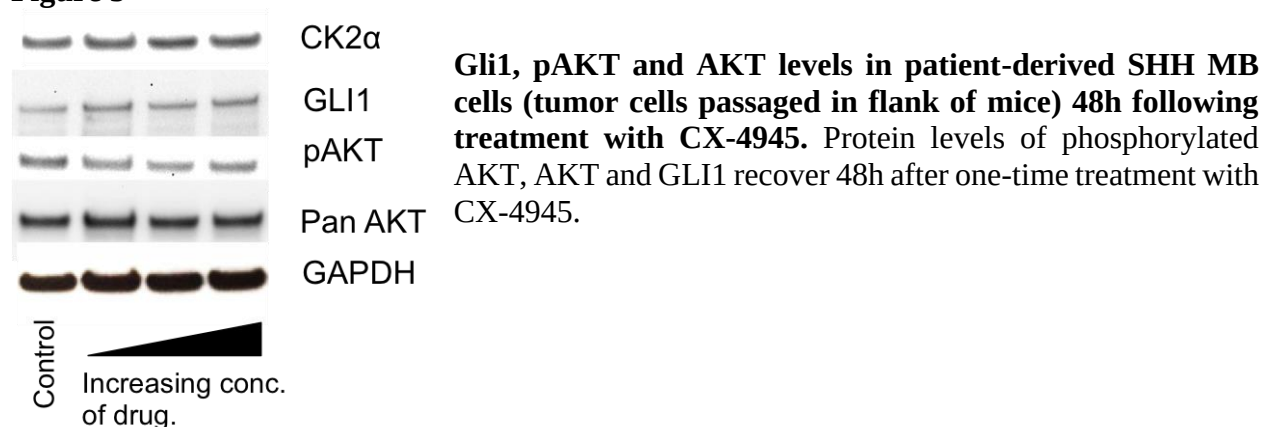


Figure 5



2.4.2 Rationale for Pharmacokinetic Studies

Plasma pharmacokinetic studies will be performed in all patients enrolled on the Phase I part of this trial and is optional for the Phase II participants. Because the pharmacokinetics of this CX-4945 are unknown in the pediatric population, these data will be essential for evaluating toxicity and disease response and for refining dosing in future clinical trials of the drug. The results of these studies will provide the basis for assessing the relationship between the disposition of CX-4945 in adults and children. Lastly, insights into the biologically active dosage will be gained by

relating CX-4945 systemic exposure (e.g., AUC) to results of pharmacodynamic studies (e.g., disease response).

2.4.2.1 *Clinical Pharmacokinetic Data*

In a Phase I study of CX-4945 in adult patients with advanced solid tumors, multiple myeloma, or Castleman's disease, the drug was administered orally as a single agent 21 days of a 28-day cycle. CX-4945 disposition was assessed on 2 dosing schedules, twice daily (BID) with pharmacokinetic studies on Days 1 and 21, and four times a day (QID) with a study on Day 8. For the BID schedule, the neat API formulation was administered as a powder in a capsule (PIC) for all dose levels except cohort 11, which received CX-4945 as an amorphous spray dried dispersion (SDD) in oral capsules (200 mg BID; 400 mg/day). The CX-4945 concentration-time data from this study were analyzed using noncompartmental pharmacokinetic methods and were summarized using descriptive statistics.²⁹

On day 1 of the BID dosing schedule, the median time of maximum (T_{max}) CX-4945 concentration ranged from 0.5 to 4 hours. The median maximum (C_{max}) CX-4945 concentration values were 123, 394, 1180, 403, 497, and 700 ng/ml for CX-4945 doses of 90, 160, 300, 460, 700, and 1000 mg, respectively. Corresponding median area under the concentration-time curves (AUC₀₋₈) values were 231, 917, 1940, 997, 1380, and 3000 ng/ml*hr for the respective CX-4945 doses. The exposure parameters (C_{max} and AUC₀₋₈) increased as the CX-4945 dose increased from 90 to 300 mg, but not at higher doses (480 to 1000 mg) most likely due to small patient numbers and wide interpatient variability.²⁹

On day 21, the median T_{max} ranged from 1 to 2 hours and the median C_{max} values were 264, 155, 810, 429, 2050, and 1450 ng/ml for CX-4945 doses of 90, 160, 300, 460, 700, and 1000 mg, respectively. Similarly, the CX-4945 AUC₀₋₈ values increased with dose and were higher than those of Day 1 except for the 160 mg dose. The median CX-4945 half-life was estimated from the accumulation ratio (AR) on day 21 and ranged from 9 to 10 hours.²⁹

In general, the CX-4945 exposure parameters at similar doses were higher after dosing with the PIC formulation than the SDD in oral capsules.

More recently, a new formulation for CX-4945 has been developed using hard shell gelatin capsules (Capsugels), known as the automated fill capsule (AFC), and will be used in the present study. A pharmacokinetic study was conducted in 36 healthy male volunteers to evaluate the bioavailability of the AFC form with regards to the PIC form at a dose of 200 mg CX-4945 under fasted conditions.⁴⁰ Specific pharmacokinetic parameters (i.e., maximum plasma concentration C_{max}, and area under the concentration curve AUC) were statistically compared using ANOVA analysis. The results of the study showed that the two formulations were near equivalent under fasted conditions.

3.0 PATIENT SELECTION

All subjects must meet the following inclusion and exclusion criteria. No exceptions will be given. Imaging studies to establish eligibility must be done within 3 weeks prior to enrollment. All other clinical evaluations to establish eligibility must be done within 7 days prior to enrollment.

3.1 Screening Criteria

3.1.1 Tumor

Patient must have a diagnosis of medulloblastoma that is recurrent or progressive. All tumors must have histologic verification either at the time of diagnosis or recurrence.

3.1.2 Adequate Pre-trial Tumor Tissue

Patient must have adequate pre-trial tumor material available for subgrouping.

3.1.3 Prior Therapy

Patient must have received and failed standard therapy for medulloblastoma.

3.1.4 Screening Consent

Participant is willing to sign a screening consent. The screening consent is for subgroup testing for all participants and for bone age determination in subjects ≤ 18 years. The screening consent is to be obtained according to institutional guidelines. Assent, when appropriate, will be obtained according to institutional guidelines.

3.2 Eligibility Criteria for Enrollment

3.2.1 Phase I Skeletally-Immature

3.2.1.1 *Skeletal maturity*

Patient must be skeletally-immature at the time of study enrollment, defined as females with a bone age < 14 years and males with a bone age < 16 years.

3.2.1.2 *Disease Status*

Patients who participate in the expansion cohort must have bi-dimensionally measurable disease, defined as at least one lesion that can be accurately measured in at least two dimensions ([Section 12.2.2](#)). Patients with measurable extraneural disease only are also eligible.

3.2.1.3 *Age*

Patient must be ≥ 3 and ≤ 18 years of age at the time of enrollment.

3.2.1.4 *BSA*

Patients enrolled on the Phase I study must have a BSA as noted below:

Dose Level	Minimum	Maximum
0 (400 mg/m ² BID)	0.84m ²	2.25m ²
1 (600 mg/m ² BID)	0.60m ²	2.00m ²
2 (800 mg/m ² BID)	0.63m ²	2.00m ²

3.2.2 Phase II

3.2.2.1 *Skeletal Maturity*

Patients must be skeletally-mature at the time of study enrollment, defined as females with a bone age ≥ 14 years and males with a bone age ≥ 16 years OR have a chronologic age > 18 years.

3.2.2.2 *Disease Status*

Patients must have bi-dimensionally measurable disease, defined as at least one lesion that can be accurately measured in at least two dimensions ([Section 12.2.2](#)). Patients with measurable extraneural disease only are also eligible.

3.2.3 Surgical Study

3.2.3.1 *Disease Status*

Surgical resection of CNS disease must be clinically indicated.

3.2.3.2 *Age*

Patient must be ≥ 3 at the time of enrollment.

3.2.3.3 *Therapy Prior to Resection*

Patients must be amenable to receiving CX-4945 for 5-7 days prior to their resection.

3.2.4 Inclusion Criteria – All Phases

3.2.4.1 *Tumor*

Patient must have a diagnosis of SHH medulloblastoma that is recurrent or progressive, confirmed histologically and by CLIA-certified methylation-based subgroup testing at Memorial Sloan Kettering Cancer Center (MSKCC), National Cancer Institute (NCI), or Nationwide Children's Hospital (NCH); see [Section 5.2](#). Results from prior testing at these designated sites using the same approaches described for this study will be accepted.

3.2.4.2 *Prior Therapy*

Patients must have received prior disease directed therapy and must have recovered from the acute treatment related toxicities (defined as $\leq$ grade 1 if not otherwise defined in eligibility criteria) prior to entering this study.

3.2.4.2.1 *Chemotherapy*

Patients must have received their last dose of known myelosuppressive anticancer therapy at least 21 days prior to enrollment or at least 42 days of nitrosourea.

3.2.4.2.2 *Investigational/Biologic Agent or Targeted therapies (non-myelosuppressive cancer therapy):*

- Biologic or investigational agent (anti-neoplastic):

Patient must have recovered from any acute toxicity potentially related to the agent and received their last dose of the investigational or biologic agent ≥ 7 days prior to study enrollment.

- For agents that have known adverse events occurring beyond 7 days after administration, this period must be extended beyond the time during which adverse events are known to occur.

- Monoclonal Antibodies and agents with known prolonged half-lives:

Patient must have recovered from any acute toxicity potentially related to the agent and received their last dose of the agent ≥ 28 days prior to study enrollment.

3.2.4.3 *Radiation*

Patients must have had their last fraction of:

- Craniospinal irradiation or total body irradiation or radiation to $\geq 50\%$ of pelvis > 12 weeks prior to enrollment.
- Focal irradiation > 4 weeks prior to enrollment

3.2.4.4 *Stem Cell Transplant*

Patient must be:

- ≥ 24 weeks since allogeneic stem cell transplant prior to enrollment with no evidence of active graft vs. host disease
- ≥ 12 weeks since autologous stem cell transplant prior to enrollment

3.2.4.5 *Growth Factors*

Patients must be off all colony- forming growth factor(s) for at least 1 week prior to enrollment (e.g., filgrastim, sargramostim or erythropoietin). 2 weeks must have elapsed if patients received long-acting formulations.

3.2.4.6 *Neurologic Status*

- Patients with neurological deficits should have deficits that are stable for a minimum of 1 week prior to enrollment, documented by a detailed neurological exam.
- Patients with seizure disorders may be enrolled if seizures are well controlled.

3.2.4.7 *Performance Status*

Karnofsky Performance Scale (KPS for > 16 years of age) or Lansky Performance Score (LPS for ≤ 16 years of age) assessed within two weeks of enrollment must be ≥ 50 . Patients who are unable to walk because of neurologic deficits, but who are up in a wheelchair, will be considered ambulatory for the purpose of assessing the performance score.

3.2.4.8 *Organ Function*

Patients must have adequate organ and marrow function as defined below:

- Absolute neutrophil count $\geq 1.0 \times 10^9$ cells/ L
- Platelets $\geq 100 \times 10^9$ cells/ L (unsupported, defined as no platelet transfusion within 7 days)
- Hemoglobin ≥ 8 g/dl (may receive transfusions)
- Total bilirubin ≤ 1.5 times institutional upper limit of normal (ULN)
- ALT(SGPT) ≤ 3 x institutional upper limit of normal (ULN)
- AST(SGOT) ≤ 3 x institutional upper limit of normal (ULN)
- Albumin ≥ 2 g/dl
- Serum electrolytes (Sodium, Potassium, Chloride) within institutional limits of normal (patients can be on enteral supplementation)

- Serum creatinine based on age/gender as noted in Table 2. Patients that do not meet the criteria in Table 2 but have a 24-hour Creatinine Clearance or GFR (radioisotope or iothalamate) ≥ 70 mL/min/1.73 m² are eligible.

Table 2:
Serum Creatinine for age/gender

Age	Maximum Serum Creatinine (mg/dL)	
	Male	Female
3 to < 6 years	0.8	0.8
6 to < 10 years	1	1
10 to < 13 years	1.2	1.2
13 to < 16 years	1.5	1.4
≥ 16 years	1.7	1.4
The threshold creatinine values in this Table were derived from the Schwartz formula for estimating GFR (Schwartz et al. J. Peds, 106:522, 1985) utilizing child length and stature data published by the CDC.		

3.2.4.9 Corticosteroids

Patients who are receiving dexamethasone must be on a stable or decreasing dose for at least 1 week prior to enrollment.

3.2.4.10 Pregnancy Prevention

Patients of childbearing or child fathering potential must be willing to use a medically acceptable form of birth control, which includes abstinence, while being treated on this study and for 12 weeks after drug cessation.

3.2.4.11 Informed Consent

Ability to understand and the willingness to sign a written informed consent document. Legally authorized representatives may sign and give informed consent on behalf of study participants.

3.3 Exclusion Criteria – All Phases

3.3.1 Pregnancy

Pregnant women are excluded from this study. Female patients of childbearing potential must have a negative serum or urine pregnancy test. If the urine test is positive or cannot be confirmed as negative, a serum pregnancy test will be required.

3.3.2 Lactation Status

Lactating females are not eligible unless they have agreed to not breastfeed their infants. There is an unknown but potential risk for adverse events in nursing infants secondary to treatment of the mother with CX-4945.

3.3.3 Concurrent Illness

- Patients with a history of any other malignancy, except patients with a secondary brain tumor if the patient's prior malignancy has been in remission for at least 5 years from the end of treatment.

- Patients with any of the following gastrointestinal disorders:
 - Difficulty with swallowing or active malabsorption (e.g., short gut) syndrome
 - Uncontrolled diarrhea (excess of 2-3 stools/day above normal frequency)
 - Gastritis, ulcerative colitis, Crohn's disease, or hemorrhagic coloproctitis
 - History of gastric or small bowel surgery involving any extent of gastric or small bowel resection
- Patients with any clinically significant unrelated systemic illness (serious infections or significant cardiac, pulmonary, hepatic or other organ dysfunction), that in the opinion of the investigator would compromise the patient's ability to tolerate protocol therapy, put them at additional risk for toxicity or would interfere with the study procedures or results.

3.3.4 Cardiac

- Corrected QT (QTc) interval is > 480ms (calculated using Bazett formula)

3.3.5 Concomitant Medications

- Patients who are receiving any other anti-cancer or investigational drug therapy are ineligible.
- Patients on warfarin

3.3.6 Prisoners

Prisoners will be excluded from this study.

3.3.7 Inability to participate

Patients who in the opinion of the investigator are unwilling or unable to return for required follow-up visits or obtain follow-up studies required to assess toxicity to therapy or to adhere to drug administration plan, other study procedures, and study restrictions.

3.4 Inclusion of Women and Minorities

NIH policy requires that women and members of minority groups and their subpopulations be included in all NIH-supported biomedical and behavioral research projects involving NIH-defined clinical research unless a clear and compelling rationale and justification establishes to the satisfaction of the funding Institute & Center (IC) Director that inclusion is inappropriate with respect to the health of the subjects or the purpose of the research. Exclusion under other circumstances must be designated by the Director, NIH, upon the recommendation of an IC Director based on a compelling rationale and justification. Cost is not an acceptable reason for exclusion except when the study would duplicate data from other sources. Women of childbearing potential should not be routinely excluded from participation in clinical research. Please see <http://grants.nih.gov/grants/funding/phs398/phs398.pdf>.

The PBTC remains committed to offering participation in our trials to subjects of all races and ethnic groups.

3.5 Treatment at the Primary Institution

All experimental protocol therapy should be dispensed and all on treatment imaging studies should be obtained at a PBTC institution. Laboratory studies, excluding pharmacokinetic and biologic assays, may be performed at a CLIA certified laboratory of the investigator's choice. Imaging utilized to determine eligibility may be performed at an outside institution if all required imaging sequences are included and the study is deemed of adequate quality by the treating team. All required physical examinations, laboratory parameters need to be performed at the primary PBTC institution during the dose finding period of the protocol.

3.6 Criteria to Start Treatment

- Subjects must start therapy within seven (7) days of enrollment.
- All patients ≤ 18 years must have a standard bone age x-ray of the wrist and hand within four (4) weeks prior to starting therapy.
- Skeletally-immature subjects must have an MRI scan of the knee (right knee) within four (4) weeks prior to starting therapy.
- Laboratory values must be no older than 7 days prior to the start of therapy. If a test that is repeated post enrollment and prior to the start of therapy is outside the limits for eligibility, it must be rechecked within 48 hours prior to the start of therapy. If rechecks are still outside the limits for eligibility, the patient may not receive protocol therapy and will be considered off study.

3.7 Criteria to Start Treatment with CX-4945 Following Surgery

The following must be documented for Surgical patients during the post-surgical period when switching to Phase I or Phase II treatment prior to receiving CX-4945. If any of these criteria are not met, the patient is not eligible to start treatment. See [Section 11](#) for details of the assessments.

3.7.1 Imaging

Subjects must have intra-operative or post-operative MRI to document disease status. Imaging studies to continue on to Phase I/II treatment must be completed within four weeks prior to starting treatment.

3.7.2 CX-4945

Patient amenable to receiving the drug CX-4945, at least 2 weeks post- surgery and no later than 4 weeks post-surgery.

3.7.3 Organ functions

Laboratory parameters must meet the eligibility criteria as defined in [Section 3.2.4.8](#). Laboratory values must be no older than 7 days prior to the start of CX-4945. If a test that is repeated post-surgery and prior to the start of therapy is outside the limits for eligibility, it must be rechecked within 48 hours prior to the start of therapy. If rechecks are still outside the limits for eligibility, the patient may not receive protocol therapy and will be considered off treatment.

3.7.4 Pregnancy status

Female patients of childbearing potential must have a negative serum or urine pregnancy test.

3.7.5 Concurrent illness

Patients must have recovered from surgical toxicities and cleared by Neurosurgery to start therapy.

3.7.6 Neurological status

Patients must be neurologically stable and on stable or a decreasing dose of steroids for at least one week.

4.0 REGISTRATION PROCEDURES

4.1 Investigator and Research Associate Registration with CTEP

Food and Drug Administration (FDA) regulations require sponsors to select qualified investigators. National Cancer Institute (NCI) policy requires all individuals contributing to NCI-sponsored trials to register with their qualifications and credentials to renew their registration annually. To register, all individuals must obtain a Cancer Therapy Evaluation Program (CTEP) credentials necessary to access secure NCI Clinical Oncology Research Enterprise (CORE) systems. Investigators and clinical site staff who are significant contributors to research must register in the Registration and Credential Repository (RCR) at <https://ctepcore.nci.nih.gov/rcr/>. The RCR is a self-service online person registration application with electronic signature and document submission capability.

RCR utilizes five-person registration types.

- Investigator (IVR) — MD, DO, or international equivalent;
- Non-Physician Investigator (NPIVR) — advanced practice providers (e.g., NP or PA) or graduate level researchers (e.g., PhD);
- Associate Plus (AP) — clinical site staff (e.g., RN or CRA) with data entry access to CTSU applications such as the Roster Update Management System [RUMS], OPEN, Rave, acting as a primary site contact, or with consenting privileges;
- Associate (A) — other clinical site staff involved in the conduct of NCI-sponsored trials;
-

RCR requires the following registration documents:

Documentation Required	IVR	NPIVR	AP	A
FDA Form 1572	✓	✓		
Financial Disclosure Form	✓	✓	✓	
NCI Biosketch (education, training, employment, license, and certification)	✓	✓	✓	
GCP training	✓	✓	✓	
Agent Shipment Form (if applicable)	✓			
CV (optional)	✓	✓	✓	

IVRs and NPIVRs must list all clinical practice sites and Institutional Review Boards (IRBs) covering their practice sites in RCR to allow the following:

- Addition to a site roster;
- Selection as the treating, credit, or consenting person in OPEN;
- Ability to be named as the site-protocol Principal Investigator (PI) on the IRB approval; and
- Assignment of the Clinical Investigator (CI) role on the Delegation of Tasks Log (DTL).

In addition, all investigators acting as the Site-Protocol PI (investigator listed on the IRB approval), consenting or treating investigator in OPEN, or as the CI on the DTL must be rostered at the enrolling site with a participating organization.

Refer to the [NCI RCR](#) page on the [CTEP website](#) for additional information. For questions, please contact the **RCR Help Desk** by email at RCRHelpDesk@nih.gov.

4.2 Cancer Trials Support Unit Registration Procedures

Permission to view and download this protocol and its supporting documents is restricted and is based on person and site roster assignment housed in the Roster Maintenance application and in most cases viewable and manageable via the Roster Update Management System (RUMS) on the Cancer Trials Support Unit (CTSU) members' website. Supplemental documents are available on the PBTC-053 Protocol Webpage.

This study is supported by the NCI CTSU.

4.2.1 IRB Approval

As of March 1, 2019, all U.S.-based sites must be members of the NCI Central Institutional Review Board (NCI CIRB) in order to participate in Cancer Therapy Evaluation Program (CTEP) and Division of Cancer Prevention (DCP) studies open to the National Clinical Trials Network (NCTN) and NCI Community Oncology Research Program (NCORP) Research Bases. In addition, U.S.-based sites must accept the NCI CIRB review to activate new studies at the site after March 1, 2019. Local IRB review will continue to be accepted for studies that are not reviewed by the CIRB, or if the study was previously open at the site under the local IRB. International sites should continue to submit Research Ethics Board (REB) approval to the CTSU Regulatory Office following country-specific regulations.

Sites participating through the NCI CIRB must submit the Study Specific Worksheet (SSW) for Local Context to the CIRB using IRBManager to indicate their intent to open the study locally. The NCI CIRB's approval of the SSW is automatically communicated to the CTSU Regulatory Office, but sites are required to contact the CTSU Regulatory Office at CTSUSRegPref@cts.cocccg.org to establish site preferences for applying NCI CIRB approvals across their Signatory Network. Site preferences can be set at the network or protocol level. Questions about establishing site preferences can be addressed to the CTSU Regulatory Office by email (CTSUSRegPref@cts.cocccg.org) or calling 1-888-651-CTSU (2878).

Sites using their local IRB or REB must submit their approval to the CTSU Regulatory Office using the Regulatory Submission Portal located in the Regulatory section of the CTSU website. Acceptable documentation of local IRB/REB approval includes:

- Local IRB documentation;
- IRB-signed CTSU IRB Certification Form; and/or
- Protocol of Human Subjects Assurance Identification/IRB Certification/Declaration of Exemption Form.

In addition, the Site-Protocol Principal Investigator (PI) (i.e., the investigator on the IRB/REB approval) must meet the following criteria for the site to be able to have an Approved status following processing of the IRB/REB approval record:

- Have an active CTEP status;
- Have an active status at the site(s) on the IRB/REB approval (applies to US and Canadian sites only) and on at least one participating organization's roster;
- If using NCI CIRB, be active on the NCI CIRB roster under the applicable CIRB Signatory record;
- Include the IRB number of the IRB providing approval in the Form FDA 1572 in the RCR profile;
- List all sites on the IRB/REB approval at Practice Sites in the Form FDA 1572 in the RCR profile; and
- Have the appropriate CTEP registration type for the protocol.

4.2.2 Additional Requirements

Additional requirements to obtain an approved site registration status include:

- An active Federal Wide Assurance (FWA) number;
- An active roster affiliation with the Lead Protocol Organization (LPO) or a Participating Organization (PO);
- An active roster affiliation with the NCI CIRB roster under at least one CIRB Signatory Institution (US sites only); and
- Compliance with all applicable protocol-specific requirements (PSRs).

4.2.3 Protocol-Specific Requirements for PBTC-053 Site Registration

Upon site registration approval in the Regulatory application, the enrolling site may access Open Patient Enrollment Network (OPEN) to complete enrollments. The enrolling site will select their credentialed provider treating the subject in the OPEN credentialing screen and may need to answer additional questions related to treatment in the eligibility checklist, if applicable.

4.2.4 Downloading Site Registration Documents

Download the site registration forms from the protocol-specific page located on the CTSU members' website. Permission to view and download this protocol and its supporting documents is restricted to institutions and their associated investigators and staff on a participating roster. To view/download site registration forms:

- Log in to the CTSU members' website (<https://www.ctsu.org>);
- Click on *Protocols* in the upper left of the screen
 - Enter the protocol number in the search field at the top of the protocol tree; or
 - Click on the By Lead Organization folder to expand, then select *PBTC* and protocol number *PBTC-053*.
- Click on *Documents*, *Protocol Related Documents*, and use the *Document Type* filter and select *Site Registration* to download and complete the forms provided. (Note: For sites under the CIRB, IRB data will load automatically to the CTSU.)

4.2.5 Submitting Regulatory Documents

Submit required forms and documents to the CTSU Regulatory Office via the Regulatory Submission Portal on the CTSU members' website.

To access the Regulatory Submission Portal log in to the CTSU members' website, go to the Regulatory and select Regulatory Submission.

Institutions with patients waiting that are unable to use the Regulatory Submission Portal should alert the CTSU Regulatory Office immediately by phone or email: 1-866-651-CTSU (2878), or CTSURegHelp@cocg.org in order to receive further instruction and support.

4.2.6 Checking Site's Registration Status

Site registration status may be verified on the CTSU members' website.

- Click on *Regulatory* at the top of the screen;
- Click on *Site Registration*; and
- Enter the site's 5-character CTEP Institution Code and click on Go.
 - Additional filters are available to sort by Protocol, Registration Status, Protocol Status, and/or IRB Type.

Note: The status shown only reflects institutional compliance with site registration requirements as outlined within the protocol. It does not reflect compliance with protocol requirements for individuals participating on the protocol or the enrolling investigator's status with the NCI or their affiliated networks.

4.3 Delegation of Tasks Log (DTL)

Each site must complete a protocol-specific Delegation of Tasks Log (DTL) using the DTL application in the Delegation Log section on the CTSU members' website. The Clinical Investigator (CI) is required to review and electronically sign the DTL prior to the site receiving an approved site registration status and enrolling patients to the study. To maintain an approved site registration status the CI must re-sign the DTL at least annually and when a new version of the DTL is released; and to activate new task assignments requiring CI sign-off. Any individual at the enrolling site on a participating roster may initiate the site DTL. Once the DTL is submitted for CI approval, only the designated DTL Administrators or the CI may update the DTL. Instructions on completing the DTL are available in the Help Topics button in the DTL application and describes DTL task assignments, CI signature, and CTEP registration requirements, as well as include a Master Task List.

4.4 Patient Enrollment

4.4.1 OPEN

The Oncology Patient Enrollment Network (OPEN) is a web-based registration system available on a 24/7 basis. OPEN is integrated with CTSU regulatory and roster data and with the LPOs registration/randomization systems for retrieval of patient registration/randomization assignment.

OPEN will populate the patient enrollment data in NCI's clinical data management system, Medidata Rave.

Requirements for OPEN access:

- Active CTEP registration with credentials necessary to secure NCI/CTSU IT systems;
- To perform enrollments or request slot reservations: Must be on a LPO roster, ETCTN corresponding roster, or participating organization roster with the role of Registrar. Registrars must hold a minimum of an Associate Plus (AP) registration type;
- If a Delegation of Tasks Log (DTL) is required for the study, the registrars must hold the OPEN Registrar task on the DTL for the site; and
- Have an approved site registration for a protocol prior to patient enrollment.

To assign an Investigator (IVR) or Non-Physician Investigator (NPIVR) as the treating, crediting, consenting, or receiving investigator for a patient transfer in OPEN, the IVR or NPIVR must list the IRB number used on the site's Institutional Review Board (IRB) approval on their Form Food and Drug Administration (FDA) 1572 in Registration and Credential Repository (RCR). If a DTL is required for the study, the IVR or NPIVR must be assigned the appropriate OPEN-related tasks on the DTL.

Prior to accessing OPEN, site staff should verify the following:

- Patient has met all eligibility criteria within the protocol stated timeframes; and
- All patients have signed an appropriate consent form and Health Insurance Portability and Accountability Act (HIPAA) authorization form (if applicable).

Note: The OPEN system will provide the site with a printable confirmation of registration and treatment information. You may print this confirmation for your records.

Access OPEN at <https://open.ctsu.org> or from the OPEN link on the CTSU members' website. Further instructional information is in the OPEN section of the CTSU website at <https://www.ctsu.org> or <https://open.ctsu.org>. For any additional questions, contact the CTSU Help Desk at 1-888-823-5923 or ctsucontact@westat.com.

Patient enrollment for this study will be facilitated using the Slot Reservation System in conjunction with patient enrollment in OPEN. Prior to discussing protocol entry with the patient, all site staff must use the CTSU OPEN Slot Reservation System to ensure that a slot on the protocol is available to the patient. Once a slot reservation confirmation is obtained, site staff may then proceed to enroll the patient to this study.

All patients with recurrent or progressive medulloblastoma must be screened prior to enrollment. Patients must sign the screening consent before sending the pre-trial tumor tissue to the screening lab (NCH, MSKCC, or NCI) to confirm their SHH subgroup (see [Section 5.2](#) for requirements) prior to enrolling in the PBTC-053 study. Only patients who have a confirmed diagnosis of SHH medulloblastoma will be eligible to enroll.

4.4.2 Pre-enrollment Screening

Pre-enrollment screening will be performed for patients who are consented for screening and willing to provide pre-trial tumor material for SHH subgroup confirmation. Sites are required to make a reservation using the CTSU OPEN Slot Reservation System before they ship the samples to the screening lab for screening. Sites are to select which screening lab they are shipping the pre-trial tumor material to on the Prerequisite screen of OPEN. Screening is facilitated by having pre-enrollment screening questions completed by a site registrar in the Prerequisite screen of OPEN. The site must complete the steps up to Prerequisite to obtain the OPEN tracking number which will be used for labeling the slides. Patient enrollment will not proceed from the Prerequisite screen until the screening lab receives the tumor material, analyzes the tissue, and enters the SHH result into the PBTC ProtoLab database.

Completing the Prerequisite screen sends an automatic email to the screening lab and site registrar with information on the screening process. Once the SHH subgroup result is entered in ProtoLab by the screening lab, an automatic email will be sent to the site registrar and the study team indicating that enrollment may proceed. Based on the communicated SHH screening result, the site registrar must return to OPEN and complete the process – either to complete enrollment of a patient for treatment who has passed the screening, or to remove the reservation of the patient who failed the screening.

Sites will be notified of the results of the SHH subgroup testing within 15 business days of receipt of samples. All screened patients who are eligible for participating in the PBTC-053 study should be enrolled via OPEN within 10 days of the site notification of a patient's SHH subgroup status as stated in the email notification. For exceptional circumstances, exceptions to this timeline will be considered after discussion with the Study Chair and the OBDMC.

4.4.3 OPEN Questions?

Further instructional information on OPEN is provided on the OPEN link of the CTSU website at <https://www.ctsuo.org> or at <https://open.ctsuo.org>. For any additional questions contact the CTSU Help Desk at 1-888-823-5923 or ctsuocontact@westat.com.

5.0 BIOMARKER, CORRELATIVE, AND SPECIAL STUDIES

5.1 Biomarker Plan

5.1.1 List of Biomarker, Correlative, and Special Studies in Order of Priority

Priority	Test Name	Test Assay	Test Type and Purpose	M/O	Timing	Specimen	Quantity Needed	Laboratory*	Section ID
1	SHH subgrouping	Illumina Infinium Human Methylation 850K BeadChip array	Integral to confirm SHH medulloblastoma subgroup affiliation	Mandatory	Baseline	Tumor tissue	Recommended minimum 5-20 µm thick unstained slides + 1 H&E stained slide from the same FFPE tissue block	Jamal Benhamida, MD Memorial Sloan Kettering Cancer Center	5.2.1
							Minimum 10 total 5µm thick FFPE sections + 1 H&E stained slide from same tissue block	Kenneth Aldape, MD National Cancer Institute	
							Recommended minimum 5-10 total 10 µm thick FFPE scrolls (submit as 2 microfuge tubes each containing 3 scrolls) + 1 H&E stained slide from same tissue block -OR- 20-25 mg of fresh flash frozen tumor + 1 H&E slide from same tissue block	Catherine Cottrell, PhD Nationwide Children's Hospital	
2	Pharmacokinetics	Liquid chromatography tandem mass spectrometry	Determine CX-4945 concentration in plasma	Mandatory for Phase I, Surgical study Optional	Phase I, II: Pre-dose, 0.5, 1, 2, 4, 6, 8 (±1), and 24 (±4) hrs on C1 D1, D28.	Plasma	Phase I, II: 16 mLs. Surgical study: 1 mL/sample	CTP Logistics, St. Jude Children's Research Hospital	5.3.1

Priority	Test Name	Test Assay	Test Type and Purpose	M/O	Timing	Specimen	Quantity Needed	Laboratory*	Section ID
				for Phase II study	Surgical study: 5 samples between Day -7 to surgery				
3	Pharmacokinetics	Liquid chromatography tandem mass spectrometry	Determine CX-4945 concentration in tumor tissue	Mandatory for Surgical study	At 2 nd resection surgery	Frozen Tumor tissue	2 x 50 to 100 mg of fresh flash-frozen tumor	CTP Logistics, St. Jude Children's Research Hospital	5.3.1
4	Pharmacodynamics	Western Blot	Exploratory biomarker to determine inhibition of CK2	Mandatory for Surgical study	Previous Surgery (if available) and at 2 nd resection surgery	Frozen Tumor tissue	3 x 50 to 100mg fresh flash-frozen tumor	Dr. Gordon Li, MD Stanford University	5.3.3
4	Pharmacokinetics	Liquid chromatography tandem mass spectrometry	Determine CX-4945 concentration in CSF	Optional	variable	CSF (+ simultaneous plasma)	0.5 ml/ CSF sample; 1 mL/ plasma samples	CTP Logistics, St. Jude Children's Research Hospital	5.3.2
6	Genomics	Whole exome sequencing	Exploratory to determine molecular profile of tumors	Optional	Previous surgery (if available) and at 2 nd resection surgery (if applicable)	Tumor tissue (FFPE and Frozen (if available) or gDNA + blood	Up to 20 sections of FFPE + 1 H&E slide, and/or 20 mg of fresh frozen or 250 ng of gDNA (genomic DNA); 5 mL blood	PBTC CRB, Q. Richard Lu, PhD	5.3.4
7	Biobanking		Exploratory for future research	Optional	Baseline	Tumor tissue and Blood PBMCs	Up to 20 unstained slides + 1 H&E stained slide 2-5mls blood	PBTC CRB	5.3.5
*Site to select one individual lab from the SHH Laboratories listed. Results from prior testing at these designated sites using the same approaches described for this study will be accepted.									

5.1.2 Specimen Collection Schedule

Specimen Type	Baseline (Pre-treatment)	Course 1, Day 1	Course 1, Day 28	2nd resection surgery, if applicable
Tumor tissue, for SHH Subgrouping	X			
Plasma, for Pharmacokinetics	X	X (see Section 5.3.1.1)	X (see Section 5.3.1.1)	X (see Section 5.3.1.2)
Tumor tissue, for Pharmacokinetics (Surgical Arm only)				X
CSF, for Pharmacokinetics (plus simultaneous blood sample(s))	X (see Section 5.3.2)			
Tumor tissue, for CK2 Kinase (Surgical Arm only)	X			X
Tumor tissue (FFPE and frozen, if available or gDNA, for Genomics)	X			X
Blood for Genomics	X			
Tumor tissue, for Biobanking	X			X if available
Blood PBMCs	X			

5.2 Integral Laboratory Studies

5.2.1 SHH Pathology – Tumor Tissue

As an integral biomarker, prior to enrollment, tumor samples for all subjects will be evaluated for SHH subgroup confirmation using a CLIA-certified methylation-based assay at one of the four site-preferred screening labs. SHH subgrouping will be determined using Illumina Infinium Human Methylation 850K BeadChip array. The enrolling site should select one individual lab from the SHH Laboratories listed below. Results from prior testing at these designated sites using the same approach described for this study will be accepted.

5.2.1.1 Assay Method

- **Memorial Sloan Kettering Cancer Center (MSKCC)**

Prior to performing methylation-derived subgrouping, a board-certified pathologist will review the H&E stained slide(s) to confirm the neoplastic tissue meets the minimum specimen requirements of the assay (e.g. at least 50% tumor purity). The methylation-based subgrouping will then be performed in MSKCC's Diagnostic Molecular Pathology CLIA-certified lab using the Illumina MethylationEPIC/850k platform in conjunction with the University of Heidelberg's brain tumor classifier (see PMID 29539639 and 29967940). Please refer to [Appendix D1](#) for additional details regarding the medulloblastoma subgrouping methodology and validation materials. In brief, genomic DNA is extracted from FFPE tissue using the Chemagic DNA Tissue kit (PerkinElmer chemagen Technologie, GmbH, Baesweiler, Germany) after manual macrodissection. For each sample, 250 ng of input is used for bisulfite conversion (EZ DNA Methylation Kit; Zymo Research; catalog number D5002), followed by an FFPE restoration step using the Infinium HD FFPE DNA Restore Kit (Illumina; catalog number WG-321-1002). Samples are processed on the Infinium 850k array and scanned using the Illumina iScan, according to the manufacturer's recommended protocol. Quality control is assessed using internal control probes analyzed in Illumina Genome Studio, a review of copy number and beta-distributions for each sample, and two external controls (i.e., a positive and negative control). Medulloblastoma subgroups are reported as methylation classes with associated calibrated scores determined by the brain tumor classifier.

- **National Cancer Institute (NCI)**

Board-certified neuropathologist Kenneth Aldape, MD will provide diagnostic pathology analysis on H&E stain slides to determine that histopathology of the tumor tissue is a medulloblastoma. If confirmation is positive, the medulloblastoma methylation-derived subgrouping will be performed at the NCI's CAP/ CLIA certified laboratory using the Infinium Assay with the Illumina Methylation EPIC BeadChip platform. The NCI CAP/ CLIA certified laboratory has performed over 2,000 clinical methylation profiles on CNS tumors to date. A large fraction of these tumors are from pediatric patients and include a robust service for medulloblastoma subclassification. Please refer to [Appendix D2](#) for additional details regarding the medulloblastoma subgrouping methodology and validation materials.

- **Nationwide Children's Hospital (NCH)**

Prior to performing methylation-derived subgrouping, board-certified neuropathologist Diana Thomas, MD, PhD will provide pathology analysis on H&E stain slide(s) to confirm the neoplastic

tissue meets the minimum specimen requirements of the assay. If confirmation is positive, the medulloblastoma methylation-derived subgrouping will be performed using the Illumina Infinium MethylationEPIC 850K Microarray kit in conjunction with, the methylation profiling classifier developed by the German Cancer Research Center (DKFZ), German Consortium for Translational Cancer Research (DKTK), and University Hospital Heidelberg (<https://www.molecularneuropathology.org/mnp>. version 11b6). Among assayed medulloblastoma tumor samples, 100% accuracy was achieved in comparison to validated clinical results derived from reference laboratories. In addition, 100% concordance among assayed medulloblastoma samples was achieved among inter and intra-run reproducibility studies, as well as inter-technologist studies. Please refer to [Appendix D3](#) for additional details regarding the medulloblastoma subgrouping methodology and validation materials.

5.2.1.2 *Collection and Handling,*

The following must be submitted to one of the designated testing sites for medulloblastoma subgrouping:

- **MSKCC:**
 - Recommended minimum 5-20 total 10µm thick unstained slides with one H&E stained slide prepared from the same FFPE tissue block.
 - Tumor content of submitted tissue should be $\geq 50\%$. Lower tumor contents may lead to indeterminate subgrouping results.
- **NCI:**
 - Recommended minimum 10 total 5µm thick FFPE sections with one 1 H&E stained slide from same tissue block
 - Tumor content of submitted tissue should be $\geq 50\%$. Lower tumor contents may lead to indeterminate subgrouping results.
- **NCH:**
 - Recommended minimum 5-10 total 10µm thick FFPE tissue scrolls (submit as 2 microfuge tubes each containing 3 scrolls) + one H & E stained slide prepared from the same block -OR-
 - 20-25 mg of fresh flash-frozen tumor + one H&E stained slide
 - It is highly recommended that tumor content be $\geq 60\%$. Lower tumor contents may lead to indeterminate subgrouping results or delays in subgrouping.

5.2.1.3 *Shipping of Specimens*

Slides and/or microfuge tubes must be labeled with the PBTC enrollment tracking number (OPEN tracking #) and protocol number. All specimens should be shipped at room temperature, except for fresh frozen samples, which should ship on dry ice to one of the designated testing sites (MSKCC, NCI, or NCH) listed on the PBTC-053 Protocol Webpage with the completed specimen transmittal form.

Slides must be shipped to testing sites within 7 – 14 days of reservation. Sites risk loss of slot reservation if slides are not shipped within the allotted timeframe. Samples should be shipped

Monday through Thursday using the PBTC-053 FedEx account. Weekend deliveries are not permitted. Sites should e-mail the individuals below or the lab representative noted on the transmittal form, if different, prior to shipping a sample:

- **MSKCC:** Jasmin Patel (patelj2@mskcc.org) and Joliza Mateo (mateoj@mskcc.org)
- **NCI:** Zied Abdullaev (zied.abdullaev@nih.gov), Kenneth Aldape (kenneth.aldape@nih.gov) and NIHMethylationProfiling@mail.nih.gov
- **NCH:** Catherine Cottrell (catherine.cottrell@nationwidechildrens.org), Jessica Scholl (jessica.scholl@nationwidechildrens.org), and Diana Thomas (diana.thomas@nationwidechildrens.org)

Sites will be notified of the results of the SHH testing within 15 business days of receipt of samples via email. Additionally, a SHH subtyping report detailing the screening results will be provided via fax to the fax number specified by the site on the specimen transmittal form. All screened patients who are eligible for participating in the PBTC-053 study must be enrolled via OPEN within 10 days of the site notification of a patient's SHH confirmation. The sample shipping date and tracking number must be documented in OPEN by the sites at the time of pre-enrollment.

5.3 Pharmacokinetic (PK) & Exploratory/Ancillary Correlative Studies

5.3.1 Pharmacokinetics – Plasma & Tumor Tissue

Mandatory PK studies for CX-4945 will be performed on all patients enrolled on the Phase I components of this trial (including surgical patients who are eligible to initiate Phase I treatment) on Days 1 and 28 of Course 1. PK studies for CX-4945 will be performed on all consenting patients enrolled on the Phase II component of this trial. The pharmacokinetics of this agent are unknown in the setting of pediatric recurrent or refractory SHH medulloblastoma.

Sites expecting to enroll a patient on PBTC-053 should request a PK kit by emailing the Center for Translational Pharmacology (CTP) at CTPLogistics@stjude.org. Please allow at least 2-3 business days for the kit to arrive.

Please refer to the Pharmacology Operations Manual (POM) on the PBTC-053 Protocol Webpage for more detailed information regarding the collection, processing, and shipment of PK samples for this study.

5.3.1.1 Sampling Strategy for Phase I Pharmacokinetic Study

On Day 1 of Course 1, serial blood samples for CX-4945 PK studies will be collected at the following times: pre-dose, 0.5, 1, 2, 4, 6, 8 (± 1), and 24 (± 4) hours after the oral dose. **The second dose of CX-4945 on Day 1 will not be given to allow collection of the 24hr timepoint.** The 24-hour sample must be collected prior to dose administration on Day 2 of Course 1.

On Day 28 (± 2) of Course 1, serial blood samples for CX-4945 PK studies will be collected at the following times: pre-dose, 0.5, 1, 2, 4, 6, 8 (± 1), and 24 (± 4) hours after the oral dose. **The second dose of CX-4945 on Day 28 will not be given to allow collection of the 24hr timepoint.** The 24-hour sample must be collected prior to the dose administration on Day 1 of Course 2.

For children less than 13 kg, contact Dr. Clinton Stewart or his designee (901-595-2400) for a sampling strategy with reduced blood requirements.

5.3.1.2 *Sampling Strategy for CX-4945 Surgical Study*

Mandatory plasma and fresh frozen tumor tissue will be obtained from all patients enrolled on this component of the clinical trial after continuous CX-4945 therapy of 5-7 days to determine the CX-4945 plasma and brain tumor concentrations.

During 5-7 days of continuous CX-4945 dosing, 1 ml whole blood sample will be collected into a labeled purple-top K₂EDTA vacutainer tube at the following times:

- Prior to starting CX-4945 (Day -5 or Day -7)
- Prior to dose on Day -3 (± 1 day)
- Prior to dose on Day -1 (± 1 day)
- On the day of surgery (Day 0 – prior to surgery)
- On the day of surgery (Day 0 – during surgery at the same time tumor tissue is collected)

Record the exact time of the day the sample is acquired and the time of the last dose of CX-4945 on the Surgical Study CX-4945 Pharmacokinetic Data Collection Form (located in the POM).

During the surgical procedure, brain tumor tissue (50-100 mg) will be acquired at the time of tumor resection. Record the time and day of sample acquisition, the anatomical location from which the tumor sample was acquired, and the condition of the sample at the time of acquisition (e.g., viable tumor material, necrotic tumor material) on the Surgical Study CX-4945 Pharmacokinetics Data Collection Form (located in the POM). Each separate piece of tumor tissue should be placed into an appropriately labeled tube.

5.3.1.3 *Sampling Strategy for Phase II Pharmacokinetic Study*

In consenting patients, on Day 1 of Course 1, serial blood samples for CX-4945 PK studies will be collected at the following times: pre-dose, 0.5, 1, 2, 4, 6, 8 (± 1), and 24 (± 4) hours after the oral dose. **The second dose of CX-4945 on Day 1 will not be given to allow the 24hr PK timepoint collection.** The 24-hour sample must be collected prior to dose administration on Day 2 of Course 1.

On Day 28 (± 2) of Course 1, serial blood samples for CX-4945 pharmacokinetic studies will be collected at the following times: pre-dose, 0.5, 1, 2, 4, 6, 8 (± 1), and 24 (± 4) hours after the oral dose. **The second dose of CX-4945 on Day 28 will not be given to allow the 24hr PK timepoint collection.** The 24-hour sample must be collected prior to the dose administration on Day 1 of Course 2.

5.3.1.4 *Plasma and Tumor Sample Collection and Processing Instructions*

- Plasma sample collection and processing instructions:

At each time point, 1 ml of blood will be collected into appropriately labeled tubes containing K₂EDTA. The sample should be inverted several times to mix and immediately placed at 4°C (wet ice or refrigerator) until it can be centrifuged to plasma. Within 1 hour of collection, the whole blood should be centrifuged at room temperature for 10 minutes at 1,000-1,300 x *g*. (see vendor specific rotor instructions for rpm to rcf conversion). The plasma supernatant should then immediately be transferred to an appropriately labeled, screw top tube and stored at -80°C within 1 hour of sample collection. Record the exact time that the sample is collected along with the exact time that the drug is administered on the Course 1 Day 1 and Course 1 Day 28 CX-4945 Pharmacokinetic Data Collection Forms that are available in the POM on the PBTC-053 webpage.

Please refer to the POM for detailed information regarding the collection, processing, and shipment of PK samples for this study.

- Tumor sample collection and processing instructions for surgical study:
Approximately 50 to 100 mg of fresh flash-frozen tumor material will be collected into an appropriately labeled 15 mL conical tube. Record the exact time and day of sample acquisition, the anatomical location from which the tumor sample was acquired, and the condition of the sample at the time of acquisition (e.g., viable tumor material, necrotic tumor material) onto the Course 1 Day 1 and Course 1 Day 28 CX-4945 Pharmacokinetics Data Collection Forms (located in the POM). Record the sample collection and shipping information in the respective eCRF in Rave. All samples (e.g., plasma, CSF, and tumor) must be shipped on dry ice.

5.3.1.5 *Shipping of Specimens*

Samples should be shipped via Priority Overnight to ensure delivery Tuesday through Friday. Shipments should be packed with a generous amount of dry ice enclosed to safeguard against shipping delays. Weekend and holiday deliveries must be avoided.

In order for sites to receive cost reimbursement, PK samples must be shipped to the CTP within 30 days of the collection of the last sample. The site should use the PBTC-053 PK FedEx account details posted on the PBTC-053 Protocol Webpage. Ship all PK samples on dry ice, along with the completed Course 1 Day 1 and Course 1 Day 28 CX-4945 Pharmacokinetics Data Collection Forms that are available in the POM on the PBTC-053 Protocol Webpage.

Sites should contact the CTP at St. Jude Children's Research Hospital at CTPLogistics@stjude.org when samples are shipped. The sample collection and shipping dates must be documented on the eCRF in Rave.

5.3.2 Pharmacokinetics – Cerebrospinal Fluid (CSF)

PK studies for CX-4945 will be performed in all consenting participants with access to CSF who agree to participate.

Please refer to the POM for more detailed information regarding the collection, processing, and shipment of PK samples for this study.

5.3.2.1 Serial CSF Studies

In patients with external CSF access (e.g., Ommaya), ventricular CSF (0.5 ml) should be obtained in a screw-top tube pre-dose and at 1, 4, and 8 (± 1) hours after drug administration. A simultaneous 1 mL plasma sample must be obtained at the time of all CSF sample collections. The exact time that the CSF and plasma samples are obtained should be recorded along with the exact time that the drug is administered on the Serial CSF Pharmacokinetic Data Collection Form.

Note: Serial CSF samples may be collected along with the Course 1 Day 1 and Course 1 Day 28 serial plasma samples described in [Section 5.3.1](#). The exact time that the CSF and plasma samples are obtained should be recorded along with the exact time that the drug is administered on the Course 1 Day 1 and Course 1 Day 28 Serial Plasma and CSF Pharmacokinetic Data Collection Forms (located in the POM).

5.3.2.2 Single CSF Studies

A single CSF sample of 0.5 mL may be available for collection where CSF is collected as part of the best clinical practice at any time during treatment (e.g., those requiring CSF diversion or VP shunt revision during therapy, or research participants requiring shunt externalization for infection), therapy should be preferentially continued without interruption, and all attempts should be made to obtain a CSF sample. A simultaneous plasma 1 mL sample should be obtained at the time of all CSF sample collection(s). The exact time that the CSF and plasma samples are obtained should be recorded along with the exact time that the drug is administered on the Single CSF Pharmacokinetic Data Collection Form (located in the POM). **Note: Single CSF PK studies should only be performed if the patient has received a dose of CX-4945 within the past 24 hours.**

5.3.2.3 Surgical Study

A CSF sample will be obtained during a surgical procedure from a patient receiving CX-4945 (i.e., during the surgery on Day 0 with the acquisition of the tumor tissue and plasma PK samples), and the CX-4945 concentration in that sample will be determined.

5.3.2.4 Collection and Handling of Specimen(s)

- Phase I and Phase II

Collect 0.5 mL of CSF in a screw-top tube along with concomitant plasma sample. Record the exact time that the CSF and plasma samples were obtained along with the exact time of drug administration on the CSF Pharmacokinetic Data Collection Form located in the POM on the PBTC-053 Protocol Webpage. Samples must be labeled with the PBTC accession number as well as the date and exact time of collection. Samples should be stored at -80°C until shipped.

- Surgical Study

CSF will be obtained (2 mL in a screw top tube) at the time of surgery after 5-7 days of daily administration of CX-4945 (at steady state) along with concomitant plasma sample. Samples must be labeled with the PBTC accession number as well as the date and exact time of collection. Samples should be stored at -80°C until shipped.

5.3.2.5 *Shipping of Specimen(s)*

Samples should be shipped via Priority Overnight to ensure delivery Tuesday through Friday. Shipments should be packed with a generous amount of dry ice enclosed to safeguard against shipping delays. Weekend and holiday deliveries must be avoided.

In order for sites to receive cost reimbursement, PK samples must be shipped within 30 days of collection of the last sample. The site should use the PBTC-053 PK FedEx account details located on the PBTC-053 Protocol Webpage. Ship all PK samples on dry ice, along with the completed CSF Pharmacokinetics Data Collection Forms, to the address provided on the PBTC-053 Protocol Webpage.

Sites should contact the CTP at St. Jude Children's Research Hospital at CTPLogistics@stjude.org when samples are shipped. The sample collection and shipping dates must be documented on the eCRF in Rave.

5.3.2.6 *Assay Method*

Plasma, CSF, and brain tumor tissue samples will be assayed for CX-4945 PK by an gradient high performance liquid chromatography assay with tandem mass spectrometry detection as developed and fully validated in the CTP.

5.3.3 Pharmacodynamics (Surgical Study cohort only) – Tumor Tissue

As an ancillary/exploratory study, mandatory Western blot studies will be done on tumor tissue of subjects who are enrolled on the Surgical Study component of this trial, to determine activation and evaluate downstream signaling of CK2 kinase.

5.3.3.1 *Assay Method*

Biomarker studies will be used to assess whether CX-4945 effectively inhibits CK2 activity and hedgehog pathway activation in tumor tissue of patients with SHH MBs. CK2 activity will be assayed via western blot (WB), by measuring levels of phosphorylation of the well-established CK2 target AKT (S129, S473).³⁷ This biomarker was similarly used in Phase I studies of CX-4945 in solid tumors. Hedgehog pathway activation will be assessed by measuring total protein levels of the well-established terminal Hh pathway target Gli1.^{38,39} Taken together, these assays will provide insight into drug mechanism and efficacy, and may provide a predictive indices of patient response.

Preclinical *in vitro* and *in vivo* assays have demonstrated that antibodies can reliably recognize the human epitopes of pAKT (S129, S473), AKT and GLI1 in human MB cell lysate (**Figure 3**). Furthermore, treatment of SHH MB cells with CX-4945 demonstrates a robust and reproducible dose and time-dependent decrease in pAKT and Gli1 that recovers 48h following treatment (**Figure 4**, Figure 5).

Western blot is a standard technique used to visualize and quantify protein expression in tissues and cells. The Li laboratory has extensive experience with protein expression and analysis using western blot and over a decade of experience specifically in assaying CK2 target phosphorylation. There are several advantages to using WB to assay CK2 and hedgehog activity; it utilizes microgram quantities of protein and therefore obtaining adequate samples presents no added

danger/invasiveness to patients that are undergoing tumor resection; the proposed epitopes have been similarly assayed via WB in dozens of published manuscripts and in phase I clinical trials of CX-4945; available antibodies for pAKT require extensive optimization to reproducibly quantify via IHC but can be reliably quantified via WB.

A priori sample size estimate for biomarker assays were done by assessing results from *in vivo* preclinical studies of mice with cerebellar SHH MB treated for 72h with CX-4945. CX-4945 resulted in an 84% decrease in CK2 activity. The overall effect size (Cohen's d) of CX-4945 treatment was 6.26131 (control od: 0.6548, stdev=0.01, treatment od: 0.5558, stdev=0.02). We conservatively predicted that in humans CK2 inhibitors would have an effect size of at least 3.13 (assuming 50% efficacy in humans compared to mice). Therefore, if a $\alpha = 0.05$, power=0.85, 4 patients are required to demonstrate efficacy. This is well within our estimated accrual.

For additional information, see [Section 2.4.1.2](#).

5.3.3.2 *Collection and Handling of Specimen*

Brain tumor tissue to be analyzed will be collected from:

- Archival frozen tissue sample from a pre-treatment resection surgery, if available.
- The 2nd resection surgery while enrolled on this trial. If the tissue amount is limited, then prioritize available tissue for the tumor tissue PK study.

Provide approximately 100 micrograms (pea-sized) of fresh flash-frozen tumor material, frozen in foil in liquid nitrogen and stored at -70°C until the time of shipping. Each separate piece of tumor tissue should be placed into an appropriately labeled tube. Samples should be labeled with:

- Patient PBTC Accession #
- PBTC Study #
- Collection Date
- Sample type (i.e., tissue)

For both samples, record the time and day of sample acquisition, the anatomical location from which the tumor sample was acquired, and the condition of the sample at the time of acquisition (e.g., viable tumor material, necrotic tumor material) onto the specimen transmittal form, located on the PBTC-053 website. Record the sample collection and shipping information in the respective eCRF.

5.3.3.3 *Shipping of Specimen(s)*

Fresh frozen tissue tumor material should be rapidly transferred (without thawing) from long term storage to a Styrofoam box filled with dry ice. Samples should be shipped for delivery Monday through Thursday with a generous amount of dry ice enclosed to safeguard against shipping delays. Weekend and holiday deliveries should be avoided.

The site should use the PBTC-053 CK2 Kinase shipping account details, located on the PBTC-053 Protocol Webpage. Sites should e-mail gordonli@stanford.edu or the lab representative noted

on the transmittal form, if different, prior to shipping a sample. Ship all samples with the completed specimen transmittal form to the address provided on the PBTC-053 Protocol Webpage

The sample must be shipped from the site to the respective laboratory within 30 days of the last sample collection in order to receive cost reimbursement. The sample collection and shipping dates must be documented in the eCRF in Rave.

5.3.3.4 *Site(s) Performing Correlative Study*
Gordon Li, MD
Department of Neurosurgery
Stanford University School of Medicine

5.3.4 Genomics – Tumor Tissue

As an ancillary/exploratory study, genomic analyses will be performed in consenting patients to investigate the correlation between response to CX-4945 treatment and the presence of specific genomic alterations and/ or specific disease subgroups in consenting patients. Tumor tissue in the form of frozen tissue and formalin fixed paraffin embedded (FFPE) tumor material, if available, from any previous surgical excision/biopsy of the primary tumor or recurrent tumor(s), will be studied.

Unstained slides, an H&E stained slide, and blood will be collected and processed for the PBTC CRB as described in [Section 5.3.5](#). When fresh frozen tumor sample is available, we would additionally request a minimum amount of 20 mg. In the event that genomic DNA (gDNA) has already been extracted from tumor material, this can be submitted in lieu of the fresh frozen tumor sample, and we would request a minimum of 250 ng of gDNA.

For all tissue specimens submitted for genomics analysis, an additional 5 mL of blood collected prior to treatment, as a normal control for targeted sequencing studies. The blood sample should be processed as described in [Section 5.3.5](#) and submitted to the PBTC CRB.

5.3.4.1 *Assay Method*

Methods for biology studies advance with technology and are subject to change. Samples on this trial will be analyzed by the most advanced methods available. Assays performed will be undertaken in combination with the Biology and Pathology Committee and CRB and may include: DNA and RNA-based panel sequencing, whole exome or whole genome sequencing, copy number array, transcriptome sequencing as well as proteomics analysis. After analysis, left over material will be stored at the PBTC specimen repository.

5.3.4.2 *Collection, Shipping, and Handling of Specimen*

- Tumor Tissue Samples and gDNA

Tumor samples must be labeled as described in [Section 5.3.5.3](#) and shipped to the PBTC CRB as described in [Section 5.3.5.4](#). Samples should be shipped Monday through Thursday via FedEx Priority Overnight using the FedEx account information and the specimen transmittal form, found on the PBTC-053 webpage.

Fresh frozen tumor samples should be shipped in a separate box on dry ice. Record the time and day of sample acquisition, the anatomical location from which the tumor sample was acquired, and the condition of the sample at the time of acquisition (e.g., viable tumor material, necrotic tumor material) onto the specimen transmittal form. Weekend deliveries are not permitted. The sites sending the materials must document sample collection and shipping dates in the eCRF.

- Blood Sample

Peripheral blood mononuclear cells (PBMCs) must be labeled as described in [Section 5.3.5.3](#) and shipped to the PBTC CRB as described in [Section 5.3.5.4](#). PBMCs should be shipped Monday through Thursday via FedEx Priority Overnight using the FedEx account information and transmittal form, located on the PBTC-053 webpage. Weekend deliveries are not permitted.

The sites sending the materials must document sample collection and shipping dates in the eCRF. Samples may be shipped with the fresh frozen tumor samples or in a separate box on dry ice. Sites should e-mail researchsupportbiorepository@chla.usc.edu or the lab representative on the specimen transmittal form, if different, prior to shipping a sample.

5.3.4.3 *Site(s) Performing Correlative Study*

Q. Richard Lu, PhD

Brain Tumor Center

Cincinnati Children's Hospital Medical Center

5.3.5 PBTC Central Review and Biorepository (PBTC CRB) and Exploratory Correlative Studies

The Pathology Central Review and Biorepository (PBTC CRB)'s function is to collect, distribute and store specimens for central pathology review and planned correlative studies which support the laboratory objectives of this protocol. Samples submitted to the PBTC CRB may be used for planned correlative studies in consenting patients. **If a patient consents to both the PBTC CRB and planned correlative studies that use the PBTC CRB, samples should be processed according to instructions in the specific correlative study section.**

The CRB will also serve as a central repository for specimens collected for future research and left over specimens (tumor tissue or blood) returned to the repository following the planned analysis from patients who consent to future research studies. These samples will be stored in the repository for undefined future studies which support the mission of the PBTC. **If the patient does not consent to future research, remaining correlative study samples will be destroyed once the PBTC-053 analysis is complete.**

5.3.5.1 *PBTC CRB Submission Guidelines*

If the patient consents to provide slides for the repository, it is recommended that the samples below be obtained within 3 months of enrollment:

- Tumor material

Slides from the original and/or recurrent surgery should be prepared for storage. The site should provide up to twenty (20) unstained sections cut at 4µm in thickness on (+) slides from the most representative section. For patients who have consented to other correlative studies (e.g., genomics, pharmacodynamics studies) on this protocol that require slides, these submitted slides will be used for the conduct of studies outlined in the corresponding sections below. Fewer unstained sections may be submitted based on size and availability of tissue. Preference is for tissue that has not previously been frozen. The corresponding pathology report (s) including immunohistochemical, special stains, and molecular/genetic results is to be uploaded to the PBTC using the secure File Upload system. These reports will be made available to the pathologist via a link in the ProtoLab.

Suitability of sections would be established by preparing one (1) H&E to ensure that the sections meet the following criteria:

- histologically representative of the reported lesion
- contain at least 60% viable tumor
- no more than 40% necrosis

- Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs may be collected by processing a 2-5 mL whole blood specimen with Ficoll or collecting the specimen in a BD Vacutainer™ CPT™ Cell Preparation Tube with Sodium Citrate or similar tube which is available locally. Once separated, all pellets must be snap frozen and stored at least at -20°C prior to dry ice shipment

5.3.5.2 Specimen Collection

➤ Processing by Ficoll tube

1. Collect 2 - 5mL of fresh blood into an EDTA tube.
2. Transfer blood into a sterile 50-mL tube and add double the amount of PBS. MIX GENTLY.
3. Set up another tube containing half of its total volume of Ficoll. For example, if there is 15 mL of blood + PBS, then use 7.5 mL of Ficoll (2:1 ratio).
4. At very slow pace (approx. 2 mL/minute), layer the blood + PBS mixture onto the Ficoll so that the solutions DO NOT MIX. Spin the blood/Ficoll at 750 g in slow mode for 30 minutes @ 25°C. After spin you will see four distinct layers: plasma (top layer), white fluffy ring (2nd layer), Ficoll (3rd layer), and blood (bottom layer).
5. Remove plasma layer down to about 1 mL above the white fluffy ring and discard.
6. Collect the entire white fluffy ring. If ring is hard to see, also take extra liquid above. Then discard everything else.
7. Place this fraction of white blood cells into a fresh 50 mL sterile tube with 20 mL of PBS. Spin down for 10 minutes @ 25°C, 750 g in fast mode. Remove the supernatant. Add back to pellet 1 mL of PBS and spin for 5 min. at 4°C at 10000rpm. Remove supernatant.
8. Freeze the pellet of WBCs in a 2ml cryovial and store at -80°C until shipment. Cryovials larger than 2 mL cannot be accepted by the PBTC CRB.
9. Please ensure that the labeling system used is designed to withstand temperatures down to -80°C. Samples should be stored at -80°C until shipment. For short term storage (2-3 weeks) -20°C is acceptable. NOTE 4°C IS NOT ACCEPTABLE STORAGE.

If it is not possible to collect the PBMC by Ficoll gradient, then separation of PBMC can be conducted using CPT tube separation as an alternative. However, the PBMC pellet **MUST BE** frozen immediately and stored at -80°C.

- Collection and Processing by CPT tube
1. Peripheral blood should be collected in a BD Vacutainer CPT™ Cell Preparation Tube with Sodium Citrate or similar tube which is available locally. 8 mL and 4 mL CPT tubes can be obtained from Fisher Scientific (Cat# 02-685-125, 02-688-81) or Becton-Dickinson (BD No.362761, 362760). The 8 mL tubes have a 6 mL minimum draw and the 4 mL tubes have a 3 mL minimum draw.
 2. Centrifuge the CPT™ tube at 1500 x g for 30 minutes at room temperature (20o C to 25o C). **DO NOT APPLY THE BREAK ON THE CENTRIFUGE.** Use acceleration 5, brake 0 (“slow mode”).
 3. It may be necessary to spin the tube longer to ensure that all of the red blood cell components have been separated from the plasma layer through the polyester gel barrier.
 4. The tube should be removed immediately from the centrifuge. The mononuclear layer and plasma lie above the polyester gel plug.
 5. Using a sterile pipette, remove as much of the plasma component (upper half of the CPT tube) without disturbing the mononuclear layer if possible and discard.
 6. Transfer mononuclear cell layer (and some residual plasma layer) to a labeled 15- mL conical centrifuge tube and add 5 mL sterile room temperature magnesium or calcium-free phosphate buffered saline (PBS) to fill the conical tube and recap.
 7. Centrifuge at 450 x g for 10 minutes at room temperature (20o C to 25o C). Use acceleration 9, brake (“fast mode”).
 8. Remove supernatant, being careful not to aspirate the cellular pellet at the bottom of the tube.
 9. Add 1 mL of sterile PBS to the pellet and gently re-suspend by pipetting up and down. Transfer the entire suspended pellet to the labeled 2ml cryovial. Cryovials larger than 2mL cannot be accepted by the PBTC CRB.
 10. Centrifuge the cryovial at 450 x g for 5 minutes (or spin down the microcentrifuge tube at 1300 x g for 5 minutes) at room temperature. Discard the supernatant. Store the cell pellet cryovial frozen at -80°C. For short term storage (2-3 weeks) -20o C is acceptable.

5.3.5.3 *Handling of Specimens*

All samples should be labeled with the following:

- Patient PBTC Accession #
- PBTC Study #
- Collection date
- Sample type
- Slide number (For slides only; designate as “PBTCR #” where the # is assigned from 1 to 20 or the highest number of unstained sections prepared sequentially, **or** “PBTCR H&E” for the H&E stained section.)

Slides, scrolls, and Formalin Fixed, Paraffin Embedded (FFPE) tumor material should be shipped at room temperature. PBMCs should be shipped with a generous amount of dry ice enclosed to safeguard shipping delays.

5.3.5.4 *Shipment of Specimens*

Refer to the PBTC CRB Specimen Transmittal Form posted on the PBTC-053 Protocol Webpage for shipping and contact information. The sample collection, shipment date, and other specimen-specific information must be documented in the eCRF in Rave. Samples collected for the repository should be shipped for delivery Monday through Thursday by completing the Internet form at <http://www.fedex.com/us/>. The FedEx user ID and password for pathology shipping can be found on the PBTC-053 Protocol Webpage. Weekend and holiday deliveries are not permitted. It is recommended that samples are shipped as soon as possible after collection.

5.3.5.5 *Pathology Central Review*

Retrospective central pathology review will be completed from the slides submitted to the PBTC CRB if the patient consents to the CRB.

Pathologist review of slides submitted to the PBTC CRB will include the following elements:

- Examination of H&E stained slides: For each subject, one (1) H&E stained slide per one representative block from the brain tumor, removed either at initial diagnosis or relapse, should be submitted for review.
- Review of the corresponding pathology report(s) of the immunohistochemical, special stains, and molecular/genetic results from current and/or original primary tumor
- If necessary, review of immunohistochemical or special stained slides. Slides submitted to the PBTC CRB will be digitized to 40X. H&E stained sections will be retained and filed at the CRB. Original immunohistochemical or special stain slides will be returned to the submitting institution.

5.4 **Neuroimaging Studies**

Patients with CNS disease will have MRI Brain with and without contrast performed prior to therapy, after courses 2, 4, 6, then every 3 courses until time of progression or completion of treatment, and at the end of treatment. MRI Spine should be performed prior to therapy and at the same time points as standard MRI Brain, if clinically indicated. Patients on the surgical study will additionally have intraoperative or post-operative (<96 hours) MRI.

5.4.1 *Imaging Guidelines*

Standard MR imaging will include Sagittal T1 MPRAGE, axial FLAIR, axial T2, post gadolinium sagittal T1 MPRAGE (with reconstructions) and axial T2 images. The standard MR parameters are listed on the PBTC NIC web page located at <http://www.childrenshospital.org/research-and-innovation/research-labs/pediatric-brain-tumor-consortium-neuroimaging-center>. There is also a link to the same PBTC-NIC webpage from the PBTC-053 webpage.

Volumetric analyses will be done at the Neuroimaging Center (NIC, Children's Hospital Boston) via the Vitrea (Vitrea™) workstation, from the axial FLAIR and T1-weighted post-contrast brain images.

5.4.2 Neuroimaging Review

Local review of MR imaging studies at each site and central review of the MR imaging studies will be conducted through the PBTC Neuroimaging Center (NIC) at study completion. NIC review will include assessment of response to therapy (as feasible). The director and one neuroradiologist of NIC will review the imaging studies. If the local and central review are not in agreement the NIC neuroradiologist will confer with the participating site to determine why there is a discrepancy via conference call.

For patients showing a partial or complete response, the following scans should be uploaded to the PBTC at the time of response:

- The baseline scan
- Scans showing a partial or complete response (PR or CR)
- The confirmation scan obtained approximately 8 weeks later (if available)
-

Once scans are uploaded, they will be electronically transferred to the PBTC Neuroimaging Center (NIC) for central review at the conclusion of the study. All patient specific data are stripped from the images and replaced with PBTC Accession numbers prior to transmitting the images to the NIC. All image data transfer is accomplished using PGP (pretty-good-privacy) 128-bit encryption which meets industry standard for secure communication.

5.5 Imaging Parameters for Patients with Extra-neural Disease

5.5.1 Computed Tomography (CT) Scans

Axial imaging of the tumor site will be performed using low-dose technique according to the ALARA (As Low As Reasonably Achievable) concept. CT slice thickness should be 5 mm or less. Imaging will be performed during the administration of intravenous contrast, whenever possible, generally at 2 mL/kg. The use of oral contrast will be determined by the individual radiologist performing the study, but may be helpful in abdominal imaging. Images will be reconstructed in soft tissue and edge-enhanced bone/lung and liver algorithms. Coronal and sagittal multiplanar reconstructions may be helpful. Serial measurements of the primary tumor will include assessment of tumor size in the same orthogonal plane at the time of each evaluation.

5.5.2 [18F]–Fluorodeoxyglucose (18FDG)-PET Scintigraphy

The patient should be fasted for at least 4 hours prior to injection of FDG. Plasma glucose should be checked and, if the patient is substantially hyperglycemic, the study should be rescheduled when adequate glucose control has been established. FDG is administered intravenously per institutional guidelines. After injection, the patient is kept at rest for 45-60 minutes and imaging is then performed. The patient should void his/her bladder immediately prior to imaging if he/she is continent of urine. Whole body imaging (including extremities) should be obtained using institutional techniques. PET may be performed in combination with CT on dual modality PET/CT scanners or in combination with MRI on dual modality PET/MR scanners. Typically, attenuation correction imaging (low-dose, non-contrast-enhanced CT, contrast-enhanced CT, or MRI) is

performed. SUV measurements are directly available on almost all PET/CT display programs using simple regions of interest.

5.6 Knee and Bone-age Imaging Studies

5.6.1 MRI of the knee

MRI is currently the standard of care for assessing musculoskeletal anatomy and development. Short tau inversion recovery images (STIR) can readily depict physeal patency. Multiple MR sequences for assessing cartilage are available and include T1-weighted spin-echo with fat saturation, and DESS (or equivalent MERGE or fat suppressed 3D spin echo) sequences. Thus, given the risk of growth plate abnormalities following treatment with CX-4945 in skeletally-immature patients, we will perform a baseline MRI without contrast of the right knee (schedule with other MRIs of the study) within 4 weeks prior to the start of therapy, after courses 2, 4, 6, then every 3 months until time of progression or completion of treatment, and at the end of treatment (if possible) to assess for side effects of this drug on the growth cartilage.

Sites are to measure the physis at 2-3 sites along its transverse plane (on the coronal and/or sagittal midline image) and record these findings longitudinally per patient.

Note: MRI of the knee does NOT require contrast, but a knee study obtained after contrast administration in conjunction with another MRI requiring contrast delivery (e.g., MRI of brain or spine) is acceptable.

MRI Sequence	Estimated Scan Time
Coronal fat saturated T1 spin echo	5.05 min per knee
Sagittal fat saturated proton density	5.34 min per knee
3D Dess	5.34 min per knee

5.6.2 Imaging Analysis

Each MRI will be coded by central imaging review (conducted using an independent reviewer). All images will be coded as follows:

- (1) Increase vs. decrease vs. stable physeal thickness
- (2) Presence or absence of cartilaginous ingrowth into the ossified metaphysis
- (3) Presence or absence of growth recovery lines (to indicate overall growth or not)

5.6.3 Bone age x-ray

Subjects ≤ 18 years must have standard bone age x-rays of the wrist and hand within four (4) weeks prior to starting therapy.

6.0 TREATMENT PLAN

Treatment will generally be administered on an outpatient basis. Reported adverse events and potential risks are described in [Section 10](#). Appropriate dose modifications are described in [Section 7](#). No investigational or commercial agents or therapies other than those described below may be administered with the intent to treat the patient's malignancy.

Timing of protocol therapy administration and response assessment studies are based on schedules derived from the experimental design or on established standards of care. Minor unavoidable departures from protocol directed therapy and/or disease evaluations for valid clinical, patient and family logistical, or facility, procedure and/or anesthesia scheduling issues are acceptable.

CX-4945 is supplied as a 200 mg hard shell gelatin capsule (Automated Fill Capsules, AFC, Capsugels) with pharmaceutically acceptable excipients. Patients will receive CX-4945 orally twice a day. Twenty-eight consecutive days will constitute one course, and subsequent courses will immediately follow, with no break in administration of the agent in the absence of toxicity. Therapy will continue for up to 26 courses (approximately 24 months).

The study will be initiated by opening 3 slots in the Phase II study at the 1000mg BID dose level or its BSA adjusted equivalent. Since the automated fill capsule (AFC) formulation has not been studied in previous clinical trials this is being done to ensure that no unexpected toxicities are observed in older subjects. If no more than 1 DLT is observed in the first 3 patients treated at the 1000mg BID dose level, enrollment on the Phase II study will continue as per the statistical design ([Section 9.1](#)) and the Phase I component of the trial will commence in skeletally-immature patients. If the initial dose level of the Phase II study is not tolerated in the first 3 patients, then the starting dose level for the Phase I study will be Dose Level 0.

For the Phase I study as well as for a subset of patients treated on the Phase II study as defined below, dosing is based on the BSA calculated at the beginning of each course of therapy. The dose prescribed should be rounded to the nearest deliverable dose based on the BSA adjustment. Dosing Tables which reflect this approach are available in [Appendix C](#). For patients older than 18 years old treated on the Phase II study as well as for patients with larger BSAs as defined in [Section 6.3](#), flat dosing will be used.

Patients will be provided with a Medication Diary for CX-4945, instructed in its use, and asked to bring the diary, any unused study medication and the study medication bottles with them to each appointment. The Patient Diary is available in [Appendix E](#).

CX-4945 should be taken on an empty stomach either 1 hour before or 2 hours after meals. CX-4945 capsules should be taken with water only. Patients who vomit a dose of CX-4945 should NOT be re-dosed, and anti-emetic therapy should be optimized prior to the next scheduled dose.

6.1 Agent Administration – Phase I, Skeletally-Immature

Skeletally-immature children with refractory or recurrent medulloblastoma of SHH subgroup will be enrolled on the Phase I component of this trial once it is initiated after a safety cohort of 3 subjects are treated on the skeletally-mature cohort. CX-4945 will be administered twice a day on a continuous basis. Each course will be 28 days (4 weeks) in duration and the first course will be

used as the Dose Limiting Toxicity (DLT) observation period. CX-4945 is available as a 200 mg hard shell gelatin capsules (Automated Fill Capsules, AFC, Capsugels). The starting dose will be Dose Level 1, 600 mg/m² BID which corresponds approximately to the BSA-adjusted adult recommended phase 2 dose (RP2D) of 1000 mg. Dose Level 0 is provided in case the adult RP2D is not well tolerated in skeletally-immature children.

The CX-4945 dose will be increased in an approximately 30% increment to Dose Level 2 in the subsequent cohort if Dose Level 1 is well tolerated (Table 3). No inpatient dose escalation will be permitted on the protocol. Only DLTs observed during the dose-finding period of therapy will be used to guide dose escalation. Dose escalation will be governed by the statistical design as described in [Section 9](#) of the protocol.

Once the MTD/RP2D is established, an expansion cohort for pediatric skeletally-immature patients will be opened to better describe the safety, PK and preliminary efficacy of the MTD of CX-4945 in this cohort as described in the Statistical [Section 9.1.1](#).

Table 3

Phase I Dose Escalation Schedule		
Dose Level	Dose of CX-4945	Eligible BSA Range
Level 0	400 mg/m ² twice a day	≥0.84m ² - ≤2.25m ²
Level 1*	600 mg/m ² twice a day	≥0.60m ² - ≤2.00m ²
Level 2	800 mg/m ² twice a day	≥0.63m ² - ≤2.00m ²
*Starting Dose		

6.2 Agent Administration – Surgical Study

A surgical arm will enroll patients with recurrent medulloblastoma of SHH subgroup, for whom surgical resection of tumor is clinically indicated and who are amenable to receiving CX-4945 for 5-7 days prior to their resection. Surgery will not be delayed for study purposes, and patients who require emergent surgery are not eligible for this component of the trial. Patients who enroll on the surgical arm will receive CX-4945 for 5-7 days prior to resection at the established pediatric MTD/adult RP2D, and tumor samples will be obtained at the time of their resection and assessed for CK2 signaling as well as CX-4945 concentration in order to determine the biological and pharmacokinetic activity of CX-4945. Patients should not take CX-4945 on the day of surgery. The surgical study will be initiated after the first 3 patients in the skeletally-mature cohort are treated for initial assessment of safety and if no more than 1 DLT is observed in these 3 subjects. Otherwise, the dose in the skeletally mature cohort will be reduced to Dose Level 0 and 3 additional skeletally mature patients will be treated at that dose level. If no more than 1 DLT is observed in those 3 patients, then the surgical component will be initiated at Dose Level 0.

- Skeletally-mature patients with SHH medulloblastoma will be eligible for enrollment as soon as accrual to the surgical study is initiated, and will receive the drug at 1000 mg BID or its BSA adjusted equivalent depending on age and BSA criteria as per [Section 6.3](#) as long as the initial safety data from the cohort of 3-subjects do not suggest a dose reduction.

- Skeletally-immature children will only be eligible to enroll on the surgical study once the MTD/RP2D is defined in the Phase I part of the study and will receive drug at the established MTD/RP2D dose for this cohort.

Tumor tissue will be collected after treatment with CX-4945. Following recovery from surgery (no earlier than 2 weeks and no later than 4 weeks), patients will resume CX-4945 for up to 26 courses if they meet laboratory parameters as defined in [Section 3.2.4.8](#). Patients who meet the criteria for initiation of treatment for the Phase I expansion cohort or Phase II part of the study post-surgery will contribute to the accrual goals of those components. The only exception to this rule are skeletally-mature Phase II subjects or Phase I expansion cohort subjects who do not have measurable disease post their on treatment surgery. Those patients will still be treated as part of the phase I or phase II cohorts (as appropriate) but will not be counted towards the accrual goals as they will not be assessable for the primary response objectives. They will however contribute to all other Phase I or II objectives including the primary toxicity objective.

Figure 6.



6.3 Agent Administration - Phase II

Skeletally-mature children and adults with SHH medulloblastoma will enroll on the Phase II component of this trial. CX-4945 will be administered twice a day at the adult RP2D, 1000 mg BID or at its BSA adjusted equivalent; however, the dose will be given continuously throughout 28-day courses. The patient will remain on CX-4945 for up to 26 courses. Accrual to this component will be staggered to ensure that the planned continuous dosing as well as the new formulation is safe in this population. More specifically 3 patients will be enrolled initially at Dose Level 1 and will be observed during the first course for any dose limiting toxicities. If no more than 1 patient experiences a DLT then 3 additional patients will be enrolled at Dose Level 1 and if no more than 1/6 patients experience a DLT then accrual will follow the Phase II design. If more than 1/6 patients experience a DLT during the first course, the dose of CX-4945 will be reduced to 800 mg BID or at its BSA adjusted equivalent given on a continuous basis.

Table 4

Phase II Dose Schedule – Skeletally-Mature Patients		
Dose Level	Dose of CX-4945	
	Dosing for patients ≤ 18 years AND BSA $\leq 1.74\text{m}^2$	Dosing for patients >18 years OR BSA $>1.74\text{m}^2$
Level 0	400 mg/m ² twice a day	800 mg twice a day
	Dosing for patients ≤ 18 years AND BSA $\leq 1.50\text{m}^2$	Dosing for patients >18 years OR BSA $> 1.50\text{m}^2$
Level 1 – Starting Dose	600 mg/m ² twice a day	1000 mg twice a day
See dosing nomogram in Appendix C		

6.4 Definition of Dose-Limiting Toxicity (DLT)

DLT will be defined as any of the events listed in this section that are at least possibly related to the investigational agent that occur during the dose-finding period (Course 1) regardless of expectedness.

- Any CX-4945 related adverse event during the dose finding observation period that leads to a dose reduction or results in the permanent cessation of therapy will be considered a Dose Limiting Toxicity. All dose modifications in all courses are to follow the guidelines provided in [Section 7.1](#) and [Section 7.2](#).
- Any CX-4945 related adverse event during the dose finding observation period which results in a delay of treatment > 14 days will be considered a Dose Limiting Toxicity.

6.4.1 Non-hematologic dose limiting toxicity is defined as:

- Any Grade 4 non-hematologic toxicity
- Any Grade 3 non-hematologic toxicity with the specific exception of:
 - Grade 3 nausea or Grade 3 vomiting that is controllable with anti-emetics
 - Grade 3 diarrhea that is responsive to supportive care
 - Grade 3 SGOT (AST) or SGPT (ALT) that returns to eligibility levels within 7 days of study drug interruption and that does not recur upon re-challenge with study drug
 - Grade 3 fever
 - Grade 3 infection
 - Grade 3 electrolyte abnormality responsive to enteral supplementation
- Any non-hematological toxicity that persists for ≥ 14 days and is considered sufficiently medically significant or sufficiently intolerable by patients that it requires treatment interruption

6.4.2 Hematologic dose limiting toxicity is defined as:

- Grade 3 or 4 thrombocytopenia
- Grade 4 neutropenia

Management and dose modifications associated with the above adverse events are outlined in [Section 7](#).

6.4.3 Dose-finding (for Phase I)/ Safety Assessment (for Phase II) Period

The dose finding period begins with the initial dose of CX-4945 and ends on the last day of course 1. Should there be a delay starting the subsequent course, dose finding will complete on the start date of the subsequent course.

Management and dose modifications for toxicities which occur outside of the dose-finding period should also follow guidelines in [Section 10](#). However, these will not be considered dose limiting for the purpose of dose escalation.

6.4.4 Dose Escalation/ De-escalation

Dose escalation/de-escalations will follow the Rolling-6 design as detailed in the statistical section.

6.5 Criteria to Start Subsequent Courses

A course may be repeated every 28 days if the patient has at least stable disease and has again met laboratory parameters as defined in [Section 3.2.4.8](#). If a patient does not meet these parameters at the end of the treatment course, then CX-4945 should be held until parameters meet the eligibility criteria.

6.6 General Concomitant Medication and Supportive Care Guidelines

Because there is a potential for interaction of CX-4945 with other concomitantly administered drugs, the case report form must capture the concurrent use of all other drugs, over-the-counter medications, or alternative therapies. The Principal Investigator should be alerted if the patient is taking any agent known to affect or with the potential for drug interactions. The study team should check a frequently-updated medical reference for a list of drugs to avoid or minimize the use of. [Appendix B](#) **Error! Reference source not found.** Patient Drug Information Handout and Wallet Card should be provided to patients.

6.6.1 Warfarin

Concomitant use of Warfarin is prohibited due to potential drug to drug interactions. Initial pharmacokinetic studies suggest that they result in increased exposure to the study drug.

6.6.2 Growth Factors

Routine use of growth factors (e.g., G-CSF, GM-CSF and erythropoietin) is not permitted. However, therapeutic use of G-CSF or GM-CSF in patients with serious neutropenic conditions, such as sepsis, may be considered at the investigator's discretion. Use of growth factors should be recorded in the Rave database.

6.6.3 Prophylactic treatment for persistent or recurrent drug-related symptoms

Prophylactic treatment for persistent or recurrent drug-related symptoms is strongly recommended (e.g., nausea/vomiting, electrolyte disturbances or diarrhea).

- Nausea/Vomiting

Premedication with an anti-emetic medication (preferably a 5-HT3 receptor antagonist) is mandated during treatment with CX-4945 dose.

- Diarrhea

Diarrhea is a common AE associated with CX-4945. It should be treated aggressively at first onset of symptoms with loperamide (dosing guidelines in **Table 5** if no local guidelines are available) or atropine containing medication if accompanied by abdominal cramps. Patients should be advised to drink plenty of water or rehydration fluids to avoid dehydration if diarrhea occurs. Dehydration prevention and correction of electrolyte disturbances should be practiced as per institution's SOP. Stool count and consistency should be noted in the patient diary if treatment for diarrhea is instituted.

Table 5

Weight Specific Guidelines for Therapeutic Use of Loperamide			
Weight (kg)	Initial (Loading) Loperamide dose (mg)	Subsequent daytime loperamide dose	Subsequent nighttime loperamide dose
8≤10	1	0.5mg q 3h	0.75mg q 4h
10≤20	1	1mg q 3h	1mg q 4h
20≤30	2	1mg q 3h	2mg q 4h
30≤42	2	1mg q 2h	2mg q4h
42	4	2mg q 2h	4mg q 4h

6.6.4 Steroids

Corticosteroids should be used at the lowest dose to control symptoms of edema and mass effect, and discontinued, if possible. Use of corticosteroids should be recorded in the RAVE database.

6.6.5 Anticonvulsants

Anticonvulsants drugs should be used, if indicated. Use of anticonvulsants should be recorded in the RAVE database.

6.6.6 Febrile neutropenia

Febrile neutropenia should be managed according to the local institutional guidelines. Measures include laboratory testing, blood and urine cultures, and institution of broad-spectrum antibiotics.

6.6.7 Pneumocystis jiroveci pneumonia (PJP) prophylaxis

The use of medication (e.g., Bactrim) for PJP prophylaxis in patients on chronic steroids is recommended, but is at the investigator's discretion.

6.6.8 Neurosurgical or other surgical procedures

If a neurosurgical procedure or other surgical procedure is required for a reason other than tumor progression (e.g., the onset of hydrocephalus), these procedures should be documented, but will not constitute criteria for declaring the patient "off therapy". CX-4945 should be held until the patient is clinically stable and has recovered from the acute effects of surgery.

6.6.9 Vitamin D supplementation

Because of potential cross-talk between the SHH and vitamin D pathways,⁴² vitamin D supplementation in insufficient patients (defined as 25-Hydroxyvitamin D level <30 ng/mL) is highly recommended with cholecalciferol 50,000 units weekly. 25-hydroxyvitamin D levels should be repeated every three months. For patients achieving sufficient vitamin D levels, maintenance dosing of cholecalciferol are 1,000 units daily or 5,000-10,000 units weekly.

6.6.10 Skeletal Toxicity Monitoring in Skeletally-Immature Children

The hedgehog pathway is known to play a critical role in skeletal development and even brief exposure to Hh inhibitors in juvenile mice has been shown to cause permanent defects in bone growth.³⁴ The permanent defects in bone growth observed in preclinical studies of Hh inhibitors were not observed in early phase clinical studies of vismodegib in children. However, there has since been a report of 3 patients demonstrating widespread and persistent growth plate fusion following prolonged exposure to vismodegib resulting in profound short stature and disproportionate growth.³⁵ Similar bone-related toxicities were reported in association with another SMO inhibitor, sonidegib, in 3 additional pediatric patients.³⁶ Given the concerns regarding bone toxicities with SHH pathway inhibition, baseline and serial knee MRIs will be performed in all skeletally-immature patients on this trial to monitor for bony abnormalities. Bone age X-rays will be obtained if growth plate abnormalities are observed on MRI and will be repeated as clinically indicated.

6.7 Duration of Therapy

In the absence of treatment delays due to adverse event(s), treatment may continue for 26 courses (approximately 2 years) or until an Off Treatment criteria is met.

6.7.1 On Study Data Submission Schedule

Pre-treatment, on-study and off-treatment data, as well as patient response data are to be recorded in the electronic data collection screens using the RAVE database. See the Required Data and Timetable for Submission form located on the PBTC-053 Protocol webpage for the schedule. For assistance, contact the PBTC Protocol Coordinator listed on the cover page.

6.7.2 Off Treatment Criteria

At the discontinuation of treatment, the “Off Treatment Date” is to be recorded in the eCRF and is to be consistent with the reason given for going off treatment. The “Last Treatment Date” is defined as the last date that the patient received protocol-based therapy. Date of “off treatment” must be the greatest of the date of last treatment, date of procedure, date of patient assessment, notification of patient/family decision, or decision made by the physician that resulted in the patient being taken off protocol treatment. The reason for discontinuation of treatment must be documented by the attending investigator in the medical record and recorded in the eCRF.

Patients will be considered Off Treatment for the following reasons:

- Development of unacceptable toxicity as outlined in [Section 6.4](#). See [Section 10](#) for specific reporting requirements.
- Progressive disease (PD) as described in [Section 12.3.4](#)

- Development of a medical or psychiatric illness that in the investigator's judgment renders the patient incapable of further therapy on this protocol or the treating physician determines continuation on this study is not in the patient's best interest.
- The patient, parent or legal guardian refuses further treatment on this protocol. In this case the investigator should clarify if the family also wishes to withdraw consent for continued participation for data collection purposes.
- Completion of all protocol defined treatment
- Pregnancy
- Non-compliance that in the opinion of the investigator does not allow for ongoing participation.
- Termination of the study by the sponsor
- Investigational Agent/Device manufacturer can no longer provide the study agent
- For surgical study participants, failure to meet criteria to start treatment within four (4) weeks of surgery.

Patients who are off protocol therapy must be followed until an “Off Study Criterion” is met.

6.7.3 Data Submission Schedule for Patients Off Treatment

All patients must be followed for a minimum of 30 days from last treatment date. Toxicities that are considered at least possibly related to the study treatment and are ongoing at the end of day 30 of last treatment date will be followed until resolution or return to baseline unless consent is withdrawn for further data submission or follow-up.

Patients in off-treatment follow-up will continue to be followed until 2 years after completion of treatment/ last study drug dose, for the monitoring of unexpected later developing toxicities or other morbidity and to document disease progression and survival. Data should be updated quarterly in the RAVE database. The requested follow-up data, including imaging requested in [Section 5.4](#) and [Section 5.5](#) are to be submitted quarterly in the RAVE database from the date the patient went off treatment. Data to be recorded during this interval are the following:

- Any adverse events that are possibly, probably, or definitely related to the study drug (see [Section 10](#) for specific reporting timelines)
- Dates of disease assessment and results of the associated disease assessments.
- Date of disease progression
- Date of commencement of new anticancer therapy
- Date of most recent contact
- Date of death

6.7.4 Criteria for Removal from Study

Patients who are off protocol therapy will be followed until they meet criteria for removal from study. The date and reason for the patient coming off study must be documented in the eCRF, and the Operations and Biostatistics Data Management Core (OBDMC) must be notified according to standard reporting guidelines (see [Section 13.2](#) and [Section 10](#) and the required Data and Timetable Submission located on the PBTC-053 webpage).

Ongoing AEs, or AEs that emerge after the patient is removed from protocol therapy but within 30 days of the last dose of study treatment, must be followed and reported via Rave and CTEP-

AERS (if applicable). Toxicities that are at least possibly related to the study treatment and are ongoing at the end of day 30 of last treatment date will be followed until resolution or return to baseline, whichever is longer.

Patients will be considered Off-Study for the following reasons:

- Patient determined to be ineligible.
 - If the patient was found to be ineligible after starting treatment, follow-up should continue for 30 days from the last administration of study drug.
- Patient did not initiate treatment on study.
- Parent, patient, or guardian withdraws consent for further required observations or data submission.
- Patient death while on study. The IRB, Investigational Drug Branch (IDB), Study Chair and OBDMC must be notified as per [Section 10.3](#).
- Patient has completed study follow-up which is defined as 2 years from completion of treatment.
- Patient has started another anti-cancer therapy. Follow-up must still be completed for 30 days following the last treatment date.
- Lost to follow-up
 - Sites must make every attempt to continue follow-up of patients during the study period as specified in [Section 6.7.1](#). Multiple attempts must be made and documented to obtain required study data before patient can be declared lost to follow-up. The study Protocol Coordinator and Study Chair must be notified before a subject is declared lost to follow-up.
- Progressive Disease
 - If PD occurs within less than 30 days of last treatment date then follow-up should continue until 30 days from off-treatment date or until commencement of additional anticancer therapy, whichever occurs first.
 - If the off-treatment reason is toxicity and the patient is subsequently found to have PD, then follow-up should continue for 30 days of last treatment date or until the toxicity resolves or returns to baseline, whichever is longer. If the patient commences new anticancer therapy within this specified follow-up period however, he/she will be taken off study on the date the new therapy is initiated.

6.7.5 Data Submission for Patients Off Study

No data will be collected documenting treatment or reporting events or disease status that occur subsequent to the official “Off Study” date with the exception of adverse events with an attribution of possible, probable, or definite that occur after the “Off Study” date for agents being studied under an IND ([Section 10](#)).

7.0 DOSING DELAYS/DOSE MODIFICATIONS

The study chair or co-chair must be notified of any dosage modifications, prior to the implementation of the dose modification. Refer to the dosing tables in [Appendix C](#) for dose reductions.

7.1 Dose Modifications for Hematological Toxicity

- If a patient experiences a grade ≥ 3 thrombocytopenia or grade 4 neutropenia, the treatment will be withheld. Counts should be checked twice weekly during this time. If the toxicity resolves to meet on study parameters within 14 days of drug discontinuation, the patient may resume treatment at the next lower dose level. For patients enrolled on the Phase 1 study at Dose Level 1, the dose should be reduced to Dose Level 0 as long as the subject meets the minimum and maximum BSA requirements in Table 3. Patients already receiving Dose Level 0 or who do not meet the BSA requirements at the time of dose limiting toxicity must be removed from protocol therapy. For Patients enrolled on the Phase II study the dose should be reduced to Dose Level 0 following the BSA and age requirements in

- Table 4. Doses reduced for toxicity will not be re-escalated, even if there is minimal or no toxicity with the reduced dose.
- If toxicity does not resolve to meet on study parameters within 14 days of drug discontinuation, the patient must be removed from protocol therapy.
- If a dose-limiting toxicity occurs in a patient who has resumed treatment at a reduced dose, the patient must be removed from protocol therapy.

7.2 Dose Modifications for Non-Hematological Toxicity

- There will be no dose modifications for skeletal toxicities.
- If a patient experiences a dose-limiting non-hematological toxicity, study drug should be withheld. If the toxicity resolves to meet on study parameters, or $\leq$ grade 1 if not otherwise specified in the inclusion criteria, within 14 days of drug discontinuation, the patient may resume treatment at the next lower dose level. For patients enrolled at Dose Level 1, the dose should be reduced to Dose Level 0 as long as the subject meets the minimum and maximum BSA requirements in Table 3. Patients already receiving Dose Level 0 or who do not meet the BSA requirements at the time of dose limiting toxicity must be removed from the study. For patients enrolled on the Phase II study the dose should be reduced to Dose Level 0 following the BSA and age requirements in

- Table 4. Doses reduced for toxicity will not be re-escalated, even if there is minimal or no toxicity with the reduced dose.
- If toxicity does not resolve to meet on study parameters or $\leq$ grade 1 if not otherwise specified in the inclusion criteria, within 14 days of drug discontinuation, the patient must be removed from protocol therapy.
- If a dose-limiting toxicity occurs in a patient who has resumed treatment at a reduced dose, the patient must be removed from protocol therapy.
- If a patient experiences an increased QTc interval $\geq$ Grade 2 after initiation of CX-4945 the patient should undergo additional EKGs as clinically indicated after consultation with a cardiologist. Dose modifications should be the same as noted above for other non-hematologic toxicities.

8.0 PHARMACEUTICAL INFORMATION

A list of the adverse events and potential risks associated with the investigational agent administered in this study can be found in [Section 10.1](#).

8.1 CX-4945

Chemical Name: Sodium 5-(3chlorophenylamino) benzo[c][2,6]naphthyridine-8-carboxylate

Other Names: Silmitasertib sodium

Classification: Selective ATP-competitive small-molecule inhibitor of protein kinase CK2

CAS Registry Number: 1009820-21-6

Chemical Formula: C₁₉H₁₁ClN₃NaO₂

Molecular Weight: 371.75

Mechanism of Action:

Molecular mechanism of action studies attribute the anti-proliferative and anti-tumor activity of CX-4945 to disruption of cell cycle regulation, induction of apoptosis, anti-angiogenesis, and anti-inflammatory activity.

Disruption of cell cycle regulation and cell survival is mediated via CX-4945 reduction of p21^{WAF1/CIP1} phosphorylation, induction of p27^{Kip1} and reduction of AKT phosphorylation. CX-4945 induces apoptosis via activation of caspase 3/7. Anti-angiogenic activity is mediated via CX-4945 reduction of transcriptional regulation of hypoxia-inducible factor-1 and activation of pVHL. CX-4945 suppresses inflammatory cytokines and chemokines like IL-6 and IL-8 via inhibiting activity of NF-kappaB. CX-4945 shows robust anti-tumor activity in multiple xenograft models.³⁷

CX-4945 has been shown to prevent the phosphorylation of and inactivate both XRCC1 and MDC1.

These two mediator/adaptor proteins are essential for DNA repair and require phosphorylation by CK2 for their function. This activity blocks the DNA repair response induced by gemcitabine and cisplatin and synergizes with these agents in models of ovarian cancer. Due to the high specificity of CX-4945 for CK2 and the extensive involvement of CK2 in multiple cellular processes, including DNA repair processes, CX-4945 is shown to enhance the activity of DNA damaging chemotherapy agents.⁵

Formulation: CX-4945 is delivered orally as a formulated API with the pharmaceutically accepted excipients in opaque hard-shell gelatin capsules (Automated Fill Capsules, AFC, Capsule gels) in one dose strength, 200 mg capsules.

How supplied: CX-4945 for oral administration will be supplied as 200 mg capsules. The CX-4945 capsules have two different packages, bottle and blister. For the bottle package, capsules are packaged in high-density polyethylene (HDPE) plastic bottles. Each bottle is individually heat sealed with a foil liner and capped with a white, child-resistant plastic cap with a paper liner. Bottles contain 70 capsules along with a polyester desiccant coil and a single 1-gram Sorb-IT desiccant canister (do not consume). In another kind of package, eight capsules are packed in a double-layer, child-resistant aluminum blister, with five blisters per carton. Both bottles and cartons will carry a label bearing the drug identification and conditions for storage.

Storage: Intact bottles should be stored at controlled room temperature (15-30°C or 59-86°F). CX-4945 capsules should be stored in their original containers and should be protected from moisture, freezing, bright light, and heat. Patients will be instructed to avoid storing the study drug in areas of excessive heat (e.g., vehicle glove box) or excessive moisture (e.g., bathroom medicine cabinet). Patients must also be instructed to keep the capsules in the original bottles and must not transfer the capsules to an alternate container.

Stability: The stability of the CX-4945 capsules will be monitored on an ongoing basis under ambient and accelerated conditions throughout the study.

Route of Administration:

CX-4945 is given orally. It should be taken on an empty stomach either 1 hour before or 2 hours after meals. CX-4945 capsules should be taken with water only. Patients should be advised to drink plenty of water or take hydration fluids to avoid dehydration if diarrhea occurs. Patients who vomit a dose of CX-4945 should NOT be re-dosed, and appropriate anti-emetic therapy should be implemented prior to the next scheduled dose.

8.1.1 Agent Availability and Ordering

CX-4945 capsules are provided by Senhwa Biosciences, Inc. CX-4945 may be requested by the Principal Investigator (or the authorized designee) at each participating institution. All regulatory documents, as required by the PBTC, must be current and up to date prior to requesting the study drug. The drug request form can be found on the PBTC-053 webpage.

In general, sites may order initial agent supplies when a subject is being screened for enrollment onto the study.

8.1.2 Agent Accountability

The investigator, or a responsible party designated by the investigator, must maintain a careful record of the receipt, dispensing, use and disposal of CX-4945 received from Senhwa Biosciences, Inc. using the appropriate NCI Investigational Agent (Drug) Accountability Record (DARF) available on the CTEP forms page. Store and maintain separate the Investigational Agent Accountability Records for each agent, strength, formulation, and ordering investigator on this protocol.

Any unused/used/expired study drug and containers may be destroyed according to the institutional standard operating procedure. The method and the record of destruction must be documented and maintained at the site.

8.1.3 Investigator Brochure Availability

The current versions of the Investigator Brochures (IBs) for the agents will be accessible to site investigators and research staff on the PBTC-053 webpage.

8.1.4 Useful Links and Contacts

- CTEP Forms, Templates, Documents: <http://ctep.cancer.gov/forms/>
- NCI CTEP Investigator Registration: RCRHelpDesk@nih.gov
- CTEP Identity and Access Management (IAM) account: <https://ctepcore.nci.nih.gov/iam/>
- CTEP IAM account help: ctepreghelp@ctep.nci.nih.gov

9.0 STATISTICAL CONSIDERATIONS

9.1 Study Design/Endpoints

Primary endpoints:

Phase I:

- Frequency and type of toxicity at each dose level
- Plasma PK parameters

Surgical Study:

- Intratumoral PK concentrations

Phase II:

- Frequency and type of toxicity
- Sustained objective response (PR-CR) rate

Secondary endpoints:

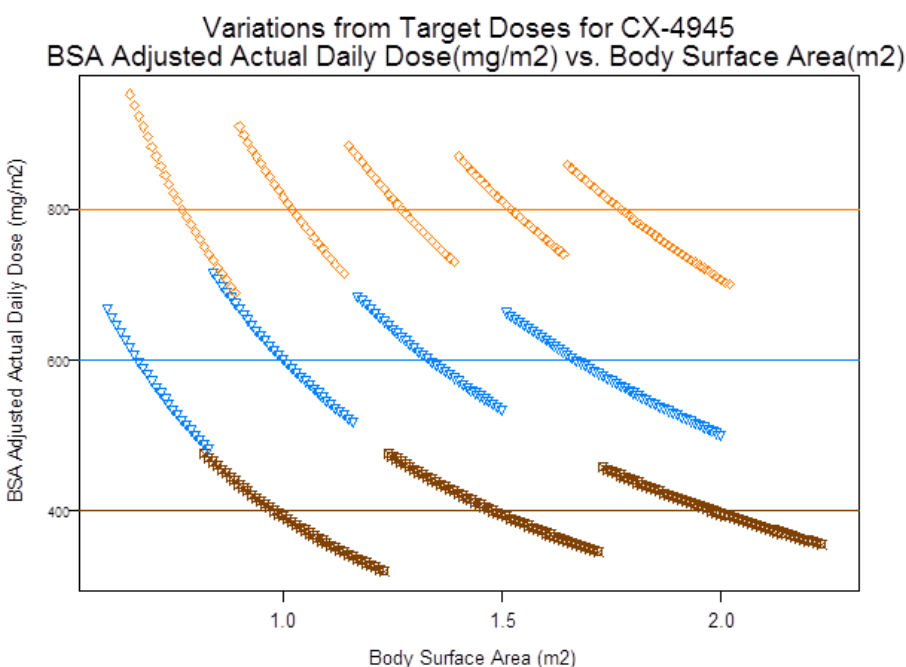
- Progression free survival
- Objective response rate in the skeletally -immature cohort
- Mean (SD) levels of Ser129-p-AKT, Ser473-p-AKT, and Gli1 in tumor tissue from patients on the surgical study arm at baseline and after treatment as well as pairwise change

9.1.1 Phase I Dose Finding for Skeletally-Immature Cohort:

This phase of the study will be initiated after 3 skeletally-mature patients are treated at the initial dose level of the Phase II study. This is being done for additional safety for the younger patients since the AFC formulation used in this study has not been previously studied. If no more than 1 DLT is observed in the first 3 patients in the skeletally-mature cohort, the Phase I study in the skeletally-immature cohort will be initiated at Dose Level 1. If more than 1 DLT is observed in the first 3 skeletally-mature subjects, the Phase I dose escalation in the skeletally-immature cohort will be initiated at Dose Level 0. [At the writing of the amendment version 4, dose finding phase in the skeletally immature cohort has been initiated at Dose Level 1.]

Skeletally-immature children with recurrent SHH medulloblastoma will be enrolled on the Phase I component of this trial. CX-4945 will be administered twice a day on a continuous basis. Each course will be 28 days (4 weeks) in duration and the first course will be used as the DLT observation period. CX-4945 is formulated as a 200 mg in hard shell gelatin capsule (Automated Fill Capsules, AFC, Capsugels). Unless suggested otherwise by the initial 3-patient safety cohort of skeletally mature subjects (see above), the starting dose will be Dose Level 1, 600 mg/m² BID which corresponds to the BSA-adjusted adult RP2D. Dose Level 0 is provided in case the adult RP2D is not well tolerated in children. The plot below displays the variations from the targeted dose levels due to the BSA adjustments as constrained by the capsule sizes. This plot suggests that the proposed dose levels are reasonable and the formulation of the agent is adequate to conduct this study in children. The overlapping segments in the plot below are accommodated by either restricting eligibility based on BSA or by slightly shifting the dosages in the dosing nomogram ([Appendix C](#)).

Figure 7.



A “Rolling-6” Phase I design will be used to estimate the MTD/RP2D, where dose escalations are planned in cohorts of two-six patients. No intra-patient escalation will be allowed.

The Rolling-6 design was recently introduced by Skolnik et al.⁴³ motivated by the observation in Lee et al.⁴⁴ that most pediatric Phase I trials in oncology have not produced excessive toxicities. A possible explanation for this could be that pediatric trials are often preceded by their adult counterparts and the knowledge gained in the latter is utilized towards ensuring safety in the former. The Rolling-6 design aims to shorten the duration of pediatric Phase I trials by minimizing the time the trial would be closed to accrual for toxicity monitoring. This is achieved by enrolling anywhere from 2 to 6 patients at a dose level without requiring that the DLT status of the patients already assigned to the same dose level are known; hence reducing the number of patients who would be turned away due to unavailability of open slots. The simulations in Skolnik et al as well as PBTC’s own simulations⁴⁵ indicate that this approach decreases the duration of a Phase I trial compared to the traditional method without increasing the overall toxicity percentage.

The Rolling-6 design allows for accrual of two to six patients concurrently onto a dose level. Decisions as to which dose level to enroll a patient are based on the number of patients currently enrolled and evaluable, the number of patients experiencing dose-limiting toxicities (DLT), and the number of patients still at risk of developing a DLT at the time of new patient entry. Dose escalation occurs if 0 out of 3-6 or at most 1 out of 6 evaluable patients experience a DLT while being treated at a dose level; otherwise, if 2 of 2-6 patients experience DLTs the dose is declared too toxic and thus above the MTD. Once a dose is determined to be too toxic, then escalation to higher dose levels is not allowed. The MTD is empirically defined as the highest dose level at which six patients have been treated with at most one patient experiencing a DLT and the next higher dose level has been determined to be too toxic. Dose escalation will begin with Dose Level 1.

9.1.2 Dose Escalation/De-Escalation Rules

The following, Table 6, which is a modified version of Table 1 from Skolnik et al⁴³, enumerates all possible scenarios and describes escalation/de-escalation rules that will be applied during dose-finding:

Table 6.

Dose Escalation/De-Escalation Rules for the Rolling-6 Design					
				Decision when Next Patient is Enrolled	
# Pats Enrolled	# Pats with DLTs	# Pats w/o DLT	# Pats with Toxicity Data Pending	Not at the Highest Dose Level	At the Highest Dose Level
2	0, 1	Any	Any	Stay	Stay
2	2	0	0	De-escalate	De-escalate
3	0	0, 1, 2	3, 2, 1	Stay	Stay
3	0	3	0	Escalate	Stay
3	1	0, 1, 2	2, 1, 0	Stay	Stay
3	≥ 2	Any	Any	De-escalate	De-escalate
4	0	0, 1, 2, 3	4, 3, 2, 1	Stay	Stay
4	0	4	0	Escalate	Stay
4	1	0, 1, 2, 3	3, 2, 1, 0	Stay	Stay
4	≥ 2	Any	Any	De-escalate	De-escalate
5	0	0, 1, 2, 3, 4	5, 4, 3, 2, 1	Stay	Stay
5	0	5	0	Escalate	Stay
5	1	0, 1, 2, 3, 4	4, 3, 2, 1, 0	Stay	Stay
5	≥ 2	Any	Any	De-escalate	De-escalate
6	0	0, 1, 2, 3, 4	6, 5, 4, 3, 2	Suspend	Suspend
6	0	5, 6	1, 0	Escalate	MTD not determined
6	1	0, 1, 2, 3, 4	5, 4, 3, 2, 1	Suspend	Suspend
6	1	5	0	Escalate	MTD not determined
6	≥ 2	Any	Any	De-escalate	De-escalate

Based on the above-outlined escalation rules, if Dose Level 0 is found to be too toxic, then the trial will be closed to accrual and the merits of amending or closing the trial permanently will be reconsidered. On the other hand, if the maximum dose level proposed for the study is deemed to be safe, then the MTD will not have been determined and consideration may be given to investigate higher dose levels. Otherwise, the highest dose level where safety was demonstrated may be recommended as the Phase II dose.

Given concerns regarding bone toxicities with SHH pathway inhibition, skeletally-immature patients will be routinely followed using serial knee MRIs (after courses 2, 4, 6, then every 3 months until time of progression or completion of treatment, and at the end of treatment). Such abnormalities regardless of grade will be monitored and will be promptly reported to the study team as well as to the PBTC toxicity committee. Any grade 3 or higher abnormalities which are at least possibly attributable to CX-4945 will also be reported to the PBTC DSMB in real time in order to obtain guidance regarding the merits of stopping therapy for the patient involved as well as for the entire cohort.

9.1.3 Expansion cohort for the Skeletally-Immature Cohort:

After determination of a pediatric MTD/RP2D based on 6 patients, an expansion cohort will be initiated to explore a preliminary signal of efficacy. We propose to enroll 10 patients with measurable disease in the CNS in total to this expansion cohort (including all patients with measurable disease in the CNS among the initial 6 patients treated at the MTD). Using an unacceptable response rate of 5% vs. desirable response rate of 30%, if we observe 2 or more objective responses in 10 patients treated at the MTD as defined in this study, this would provide adequate evidence to suggest that additional studies with this agent may be warranted in recurrent pediatric SHH medulloblastoma. Objective responses (PR and CR) must be sustained for at least 8 weeks with central radiographic confirmation. This design has attained type 1 error of 8.6% and attained power of 85% using exact binomial calculations. The additional toxicity and PK information that will be obtained from this expansion cohort will also enable us to better characterize the safety/tolerability as well as pharmacokinetics of this agent in younger patients.

9.1.4 Safety and Phase II Study in Skeletally-Mature Patients:

Skeletally-mature children and adults with SHH medulloblastoma will enroll on the Safety and Phase II component of this trial which will open in advance of the Phase I study. Accrual to this component will be staggered to ensure that the new formulation as well as the suggested continuous dosing at 1000mg BID which differs from the previous adult studies is safe in this population. More specifically initially 3 subjects will be enrolled at the 1000 mg BID dose or its BSA adjusted equivalent and will be observed during the first course for any dose limiting toxicities. If no more than one patient experiences a DLT then 3 additional skeletally-mature patients will be treated at the 1000 mg BID dose or its BSA adjusted equivalent. If no more than 1/6 patients experience a DLT at this dose level, accrual will follow the Phase II design. If more than 1 patient experiences a DLT in the first 6 patients we will de-escalate to Dose Level 0 (800mg BID or its BSA adjusted equivalent). In that scenario, we will open 6 slots at once to assess the safety of Dose Level 0.

Once safety of a dose level is established based on 6 subjects, a Simon two-stage minimax design will be used to assess the rates of objective response prior to progression of skeletally-mature patients with recurrent SHH medulloblastoma. Objective responses (PR and CR) must be sustained for at least 8 weeks with radiographic confirmation. The primary analyses will be based on all eligible patients with measurable CNS disease at baseline who receive any CX4945 and the sustained objective responses must be confirmed by central review. CX4945 will be deemed not sufficiently active if there is a lack of evidence ($\alpha=0.10$) that the true, unknown response rate is greater than 10%. The design will have 90% statistical power to detect a true, unknown response rate of 30%. In the first stage 16 patients will be accrued. If 1 or no responses are observed in these

16 patients, the study will be stopped. Otherwise, 9 additional patients will be accrued for a total sample size of 25. CX4945 will be declared promising if 5 or more responses are observed in 25 patients. **Table 7** below summarizes the decision rules based on this design:

Table 7.

Simon's Minimax Two-Stage Design ($\alpha=\beta=0.10$)
Response Rate: Unacceptable $\leq 10\%$, Desirable $\geq 30\%$

	n	Decision Rule
Stage I	16	Stop if the number of subjects with sustained objective response ≤ 1
Stage II	25	Reject if the number of subjects with sustained objective response ≤ 4

This design yields an attained type I error rate of 9.51% and power of 90.3% when the true response rate is 30%. Given the aggressive nature of this tumor, the response observation period will be the entire treatment duration as unless the CX4945 is truly effective, most patients are expected to progress relatively quickly. If at any point during the study it becomes apparent that the efficacy thresholds will not be attained whether at the interim analysis or the final analysis then accrual to this stratum will be stopped short of the planned sample size.

As of protocol version 5.0, safety of the starting dose in the skeletally mature patients has been determined and enrollment has been expanded to the Stage I sample size as indicated by Table 7 above.

Efficacy analyses of Subjects with Extraneural Disease Only:

Subjects with measurable extraneural disease only are eligible to enroll on this protocol however they will not contribute to the primary efficacy analyses described above for the subjects with measurable CNS disease as the response rates for extraneural disease are thought to be different. Thus, these subjects will be analyzed as a separate cohort for the efficacy analyses but they will contribute to the safety, PK and biology objectives in a similar fashion as the rest of the subjects. Subjects with extraneural disease only are ultra-rare and thus we do not expect to enroll any more than 6 subjects.

9.1.5 Surgical Cohort

The surgical study will be initiated after the first 3 patients in the skeletally-mature cohort are treated for initial assessment of safety and if no more than 1 subject experiences a DLT in these 3 subjects. Otherwise, the dose in the skeletally mature cohort will be reduced to Dose Level 0 and 3 additional skeletally mature patients will be treated at that dose level. If no more than 1 DLT is observed in those 3 patients then the surgical component will be initiated at Dose Level 0. [As of amendment version 4, surgical study has been initiated at Dose Level 1 in the skeletally mature patients.]

There is no specific statistical design for the surgical cohort as these subjects are relatively rare though based on previous similar studies we expect to enroll approximately 6 subjects to this cohort. Subjects who participate in the surgical study and meet the eligibility criteria to initiate treatment on the Phase I or Phase II studies post-surgery will contribute to the accrual goals of

those studies. Surgical cohort subjects who do not meet the eligibility criteria for the Phase I or Phase II studies (e.g., no-measurable disease) will still be treated at the same dose they would have received but will not count toward the accrual goals for the efficacy objectives. They will however contribute to all other study objectives including primary objectives of toxicity.

Enrollment to the surgical cohort will continue until enrollment to **both** the Phase I and II components of the study are completed. In the event that the Phase II study completes accrual and the efficacy decision is pending or preliminary efficacy is demonstrated, then a skeletally-mature surgical patient can still enroll on the study and be treated as before since this patient will contribute to the surgical component of the trial as well as to the toxicity primary objective of the Phase II study. The same will be true if the Phase I expansion component is completed and a skeletally-immature surgical subject wishes to enroll, as long as the efficacy decision in this cohort is either pending or the efficacy target has been reached.

9.2 Sample Size/Accrual Rate

9.2.1 Projected Accrual Rates and Study Duration

For the Phase I component, since the rolling 6 design allows for up to 6 patients at each dose level and given the lack of alternative treatments for these patients, we may enroll 6 patients per dose level. With the expectation that up to 3 dose levels will be studied, we anticipate maximum enrollment of 18 evaluable patients. Considering the expansion cohort which will enroll 4 additional patients at the MTD, the total expected sample size for the Phase I component is 22 evaluable patients, barring a de-escalation after the MTD/PR2D is declared based on the data from the expansion cohort.

Medulloblastomas are extremely rare in adults and most medulloblastomas occur in individuals between 20-44 years of age. (<http://rarediseases.org>) Despite their rarity, based on epidemiologic estimates, adult cases are thought to constitute 14-30% of all medulloblastomas (<http://radiopaedia.org/articles/medulloblastoma>) and it is estimated that 50-60% of all adult medulloblastomas are of the SHH subtype.^{46,47} In comparison SHH medulloblastomas constitute only 10-20% of pediatric cases. Thus, there are as many new cases of adult SHH-driven medulloblastoma diagnosed in the United States as there are pediatric SHH cases. While most of the PBTC member institutions are primarily pediatric hospitals, almost all are affiliated with adult oncology centers and can enroll adult patients on trials when available. This was demonstrated by the rapid accrual on the PBTC-025B study (32 patients in 3 years). Given that some of the pediatric patients (between 14-18 years) will also meet the eligibility criteria for skeletal maturity, we expect to enroll approximately 10-12 patients per year on this trial as was done in the PBTC 025B study and thus hope to reach the accrual goal of 25 patients in 2.5-3.5 years, barring prolonged accrual suspension for interim analysis.

We also expect to enroll no more than 6 subjects with extraneural disease only most of whom are expected to be in the skeletally mature cohort.

Although it is difficult to estimate the number of patients who will be available for the surgical cohort, our past experience with similar PBTC studies suggests approximately 4-6 such patients may be enrolled during the duration of the study. In order for patients to be considered evaluable, for the surgical component, samples from the surgical resection must be considered viable tumor

and not necrotic material by the neurosurgeon. There is no information available regarding the variability associated with intratumoral PK parameters and thus the sample size of 6 was chosen on pragmatic grounds and based on the fact that for plasma PK, dose cohorts of 3-6 patients are often used in Phase I trials. Enrollment to the surgical cohort will be possible for the skeletally-mature cohort after a safety run in based on 3 skeletally-mature subjects treated on the Phase II study whereas skeletally-immature patients will be eligible to enroll once an MTD/RP2D is established in this group. Accrual to the surgical study will cease once enrollment to both the Phase I expansion and Phase II studies are completed.

It is typical to encounter patients who enroll on a trial but turn out to be ineligible or inevaluable for various reasons. If the trial continues up to its planned maximum sample size we will enroll approximately 58 evaluable patients. With an expected 10-15% ineligible/ inevaluable patients as well as potentially a few additional patients in the surgical cohort, the maximum sample size for this trial may be up to 66 subjects. The projected accrual rates are as follows:

- Phase I Trial (skeletally-immature patients): 6-10 patients per year
- Phase II Trial (skeletally-mature patients): 10-12 patients per year
- Surgical cohort: 2-3 patients per year

Thus, we expect to complete accrual to the study in 3-4 years.

As of amendment version 4, enrollment rates are notably slower than originally projected. The provision to allow screening at multiple labs was partially motivated by the desire to increase enrollment rates. Once Amendment version 4 is activated, it is the study team's hope that accrual rates will be close to what was originally projected.

Table 8.

PLANNED ENROLLMENT REPORT

Racial Categories	Ethnic Categories				Total
	1 Not Hispanic or Latino		2 Hispanic or Latino		
	Female	Male	Female	Male	
American Indian/ Alaska Native				1	1
Asian	1	2			3
Native Hawaiian or Other Pacific Islander					0
Black or African American	2	4		1	7

Racial Categories	Ethnic Categories				Total
	1 Not Hispanic or Latino		2 Hispanic or Latino		
	Female	Male	Female	Male	
White	18	33	2	2	55
More Than One Race					
Total	21	39	2	4	66

9.3 Analysis of Primary Endpoints

Re-analyses of methylation data to confirm SHH subtype:

With amendment version 4, the study will allow screening at multiple labs using very similar, but not-identical, processes. As a post-hoc QC check, once accrual is completed, we will retrospectively obtain the raw data from the methylation arrays from all labs that perform testing on the study. We will run the Heidelberg classifier across the array data for all patients enrolled and document that they all cluster within the SHH group. In the event that this analysis suggests a discrepancy, the result will be discussed with the original lab that performed the eligibility screening in an effort to reach a consensus. Patients will not be made ineligible based on this analysis, but discrepant results will be reported to the DSMB and will be included in the associated manuscript.

9.3.1 Analysis Plan for Toxicity and Response

We will summarize toxicities observed on the study by strata, dose level and attribution in a table format. All patients who receive at least 1 dose of the study drug will be included in these analyses.

The objective response (CR+PR) rates observed in this trial will be described separately for the skeletally-mature and immature cohorts. However, as a secondary analyses, we will also provide a combined response rate estimate as well as its exact 90% confidence interval.

9.3.2 Statistical Analysis of Pharmacokinetics

Plasma drug concentrations and pharmacokinetic parameters will be presented in tabular and graphical form. Pharmacokinetic parameters of interest, such as maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), elimination half-life ($t_{1/2}$), apparent volume of distribution (V_c/F), apparent oral clearance (CL/F), and area under the plasma concentration time curve (AUC) will be estimated using non-compartmental methods for each patient. Dose proportionality in pharmacokinetic parameters will be investigated by performing one-way analysis of variance (ANOVA) on dose-normalized parameters (dose-normalized C_{max} and AUC). In addition, a population pharmacokinetic analysis of the plasma drug concentration-time data will be performed using nonlinear mixed effect modeling. The population modeling approach will allow for estimating the typical population values of the pharmacokinetic parameters and for

quantifying the inter- and intra-variability of each parameter within the patient population. A covariate analysis will be performed to explore the potential effects of patient characteristics (e.g., demographics and clinical chemistry covariates) on model parameters associated with inter-individual variability.

9.4 Analysis of Secondary Endpoints

Prolonged stable diseases as captured by progression free survival (PFS) will also be reported in a descriptive fashion using KM approaches separately for the skeletally-mature and immature cohorts treated at the MTD/RP2D. PFS is defined as the time between treatment initiation until progression or death for any reason for patients who experience an event and last follow-up for patients who are event-free at the time of analysis.

Data from blood specimens and tumors will be reported descriptively. Changes in biological markers will be also be described via plots and summary statistics. Paired tests may also be used to capture any significant changes in these markers pre and post treatment. We will also descriptively summarize any associations between molecular subgroup designations and other biology correlates with observed response to CX-4945 as well as PFS.

We will also descriptively summarize any associations between PK/PD data and other biology correlates with observed response to CX-4945 as well as PFS.

10.0 ADVERSE EVENTS: LIST AND REPORTING REQUIREMENTS

Adverse event (AE) monitoring and reporting is a routine part of every clinical trial. The following list of AEs ([Section 10.1](#)) and the characteristics of an observed AE ([Section 10.2](#) and [Section 10.3](#)) will determine whether the event requires expedited reporting via the CTEP Adverse Event Reporting System (CTEP-AERS) **in addition** to routine reporting.

- *Baseline Abnormalities*

Any baseline (pretreatment) abnormalities observed during the initial physical examination should be recorded in the case report forms (CRFs). For lab abnormalities, only those with defined CTCAE terms should be recorded unless otherwise specified below.

- *Treatment or within 30 days of treatment*

Only record adverse events Grades 1 and 2 in the eCRFs if the attribution is at least **possibly** related to CX-4945. Record all adverse events Grades 3 through 4 and deaths, regardless of attribution on the eCRFs.

10.1 Expected Adverse Events and Potential Risks List for CX-4945

The following adverse events occurred in subjects treated with CX-4945 and are to be considered expected with CX-4945 for reporting purposes. Refer to the most current version of the CX-4945 Investigator Brochure for additional adverse event information.

Table 9

MedDRA System Organ Class	Adverse Event Term	Frequency
Gastrointestinal disorders	Diarrhea	Common (> 20%)
Gastrointestinal disorders	Nausea	Common (> 20%)
Gastrointestinal disorders	Vomiting	Common (> 20%)
Metabolism and nutrition disorders	Hypokalemia	Common (> 20%)
General disorders and administration site conditions	Fatigue	Common (> 20%)
Metabolism and nutrition disorders	Hyponatremia	Occasional (4-20%)
Metabolism and nutrition disorders	Hypophosphatemia	Occasional (4-20%)
Investigations	Lymphocyte count decreased	Occasional (4-20%)
Blood and lymphatic system disorders	Anemia	Occasional (4-20%)
Musculoskeletal and connective tissue disorders	Musculoskeletal and connective tissue disorders, Other – Growth plate abnormalities Grade 3 or	Unknown

	higher	
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10.2 Adverse Event Characteristics

10.2.1 CTCAE term (AE description) and grade

The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 5.0. A copy of the CTCAE version 5.0 can be downloaded from the CTEP web site http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm.

- Growth plate abnormalities when noted on required knee MRIs are considered medically significant (Grade 3) and require both expedited and routine reporting. The event should be reported using the “Musculoskeletal and connective tissue disorders, Other, specify” term and site should specify the finding as “growth plate”.

10.2.2 For expedited reporting purposes only

Other AEs for the protocol that do not require expedited reporting are outlined in [Section 10.3.3](#).

10.2.3 Attribution of the AE:

- Definite – The AE is clearly related to the study treatment.
- Probable – The AE is likely related to the study treatment.
- Possible – The AE may be related to the study treatment.
- Unlikely – The AE is doubtfully related to the study treatment.
- Unrelated – The AE is clearly NOT related to the study treatment.

10.3 Expedited Adverse Event Reporting

Expedited AE reporting for this study must use CTEP-AERS (CTEP Adverse Event Reporting System), accessed via the CTEP Website (<https://eapps-ctep.nci.nih.gov/ctepaers>). The reporting procedures to be followed are presented in the “NCI Guidelines for Investigators: Adverse Event Reporting Requirements for DCTD (CTEP and CIP) and DCP INDs and IDEs” which can be downloaded from the CTEP Web site (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/adverse_events.htm). These requirements are briefly outlined in the tables below ([Section 10.3.2](#)).

In the rare occurrence when internet connectivity is lost, a 24-hour notification is to be made to the PBTC by telephone at 901-595-7793. Once internet connectivity is restored, the 24-hour notification phoned in must be entered electronically into CTEP-AERS by the original submitter at the site.

10.3.1 Distribution of Adverse Event Reports

CTEP-AERS is programmed for automatic electronic distribution of reports to the following individuals: Principal Investigator and the responsible Protocol Coordinator at the PBTC, the local treating physician, Senhwa Biosciences, Inc. (safety@senhwabio.com), the Reporter and Submitter. CTEP-AERS provides a copy feature for other e-mail recipients.

The following must be copied on expedited reports (24-hour notification and the complete report) submitted via CTEP-AERS:

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The IND holder, St. Jude Children's Research Hospital, shall notify the FDA of any event that is both a serious suspected adverse reaction and unexpected in accordance with FDA rules and regulations (21 CFR 312.32). Follow-up information to a safety report will be submitted, as requested.

The IND Sponsor or designee will submit a report of the unexpected, suspected adverse event to the FDA. The FDA prefers these reports to be made on a Med Watch 3500 form, but alternative formats are acceptable (e.g., a summary letter). The report should describe the event as fully as possible. Supporting documentation (lab reports, summary notes, and autopsy report) should accompany the report. A fatal or immediately life-threatening suspected adverse event will be reported to the FDA within 7 calendar days of the receipt of the initial report by the IND sponsor. A non-fatal, non-life threatening unexpected, suspected, serious adverse event will be reported to the FDA within 15 calendar days of receipt of the initial report by the IND Sponsor.

The PBTC OBDMC will post all IND Safety Letters on the PBTC-053 webpage. Sites will be notified via email of the receipt of the IND Safety Letter(s) and instructed to submit these to their local IRB in accordance with the institution's requirements.

A copy of the Annual Progress Reports is submitted by the IND holder as required by FDA, European Union (EU), Pharmaceutical and Medical Devices agency (PMDA) or other local regulators by the sponsor-investigator. This submission will be cross referenced according to local regulations to St. Jude Children’s Research Hospital and to the Senhwa Biosciences, Inc. product number at the time of submission.

10.3.2 Expedited Reporting Guidelines

Use the NCI protocol number and the protocol-specific patient ID assigned during trial registration on all reports.

Note: A death on study requires both routine and expedited reporting, regardless of causality as long as the death occurred within 30 days after the last administration of the investigational agent. Attribution to treatment or other cause must be provided.

Death due to progressive disease should be reported as Grade 5 “Disease progression” in the system organ class (SOC) “General disorders and administration site conditions.” Evidence that the death was a manifestation of underlying disease (*e.g.*, radiological changes suggesting tumor growth or progression; clinical deterioration associated with a disease process) should be submitted.

Phase 1 and Early Phase 2 Studies: Expedited Reporting Requirements for Adverse Events that Occur on Studies under an IND/IDE within 30 Days of the Last Administration of the Investigational Agent/Intervention ^{1,2}

FDA REPORTING REQUIREMENTS FOR SERIOUS ADVERSE EVENTS (21 CFR Part 312) NOTE: Investigators <u>MUST</u> immediately report to the sponsor (NCI) <u>ANY</u> serious adverse events (SAEs), whether or not they are considered related to the investigational agent(s)/intervention (21 CFR 312.64). An AE is considered serious if it results in <u>ANY</u> of the following outcomes: 1) Death 2) A life-threatening AE 3) An AE that results in inpatient hospitalization or prolongation of existing hospitalization for ≥ 24 hours. 4) A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions 5) A congenital anomaly/birth defect. 6) Important Medical Events (IME) that may not result in death, be life threatening, or require hospitalization may be considered serious when, based upon medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. (FDA, 21 CFR 312.32; ICH E2A and ICH E6).	
<u>ALL SAEs</u> that meet the above criteria <u>MUST</u> be immediately reported to the NCI via CTEP-AERS within the timeframes detailed in the table below.	
Grade 1-2 Timeframes	Grade 3-5 Timeframes
24-Hour notification, 10 Calendar Days	24-Hour notification, 5 Calendar Days
NOTE: Protocol-specific exceptions to expedited reporting of SAEs are found in Section 10.3.3 . <u>Expedited AE reporting timeframes are defined as:</u>	

○ “24-Hour notification, 5 Calendar Days” - The SAE must initially be reported via CTEP-AERS within 24 hours of learning of the SAE, followed by a complete expedited report within 5 calendar days of the initial 24-hour report. ○ “24-Hour notification, 10 Calendar Days” - The SAE must initially be reported via CTEP-AERS within 24 hours of learning of the SAE, followed by a complete expedited report within 10 calendar days of the initial 24-hour report.
¹SAEs that occur more than 30 days after the last administration of investigational agent/intervention and have an attribution of possible, probable, or definite require reporting as follows: Expedited 24-Hour notifications are required for all SAEs followed by a complete report • Within 5 calendar days for Grade 3-5 SAEs • Within 10 calendar days for Grade 1-2 SAEs ²For studies using nuclear medicine or molecular imaging IND agents (NM, SPECT, or PET), the SAE reporting period is limited to 10 radioactive half-lives, rounded UP to the nearest whole day, after the agent/intervention was last administered. Footnote “1” above applies after this reporting period. Effective Date: August 30, 2024

10.3.3 Additional Protocol-Specific Expedited Adverse Event Reporting

Exclusions

For this protocol only, the AEs/grades listed below do not require expedited reporting via CTEP-AERS. However, they still must be reported through the routine reporting mechanism ([Section 10.4](#)):

CTCAE SOC	Adverse Event	Grade	≥24h Hospitalization ^a
Investigations	Lymphocyte count decreased	≤4	Regardless

^a Indicates that an adverse event required hospitalization for ≥24 hours or prolongation of hospitalization by ≥24 hours of a patient.

10.4 Routine Adverse Event Reporting

All Adverse Events must be reported in routine study data submissions to CDUS by PBTC. AEs reported expeditiously through CTEP-AERS must also be reported in routine study data submissions.

Adverse event data collection and reporting, which are required as part of every clinical trial, are done to ensure the safety of patients enrolled in the studies as well as those who will enroll in future studies using similar agents. AEs are reported in a routine manner at scheduled times during the trial using Medidata Rave. For this trial the Adverse Event CRF is used for routine AE reporting in Rave.

10.5 Pregnancy

Although not an adverse event in and of itself, pregnancy as well as its outcome must be documented via **CTEP-AERS**. In addition, the **Pregnancy Information Form** included within the NCI Guidelines for Adverse Event Reporting Requirements must be completed and submitted to CTEP. Any pregnancy occurring in a patient or patient’s partner from the time of consent to 90 days after the last dose of study drug must be reported and then followed for outcome. Newborn infants should be followed until 30 days old. Please see the “NCI Guidelines for Investigators:

Adverse Event Reporting Requirements for DCTD (CTEP and CIP) and DCP INDs and IDEs” (at http://ctep.cancer.gov/protocolDevelopment/adverse_effects.htm) for more details on how to report pregnancy and its outcome to CTEP.

10.6 Secondary Malignancy

A *secondary malignancy* is a cancer caused by treatment for a previous malignancy (*e.g.*, treatment with investigational agent/intervention, radiation or chemotherapy). A secondary malignancy is not considered a metastasis of the initial neoplasm.

CTEP requires all secondary malignancies that occur following treatment with an agent under an NCI IND/IDE be reported expeditiously via CTEP-AERS. Three options are available to describe the event:

- Leukemia secondary to oncology chemotherapy such as acute myelocytic leukemia (AML)
- Myelodysplastic syndrome (MDS)
- Treatment-related secondary malignancy

Any malignancy possibly related to cancer treatment (including AML/MDS) should also be reported via the routine reporting mechanisms outlined in each protocol. The Adverse Event eCRF should be updated with the appropriate description within 1 week of learning of the secondary malignancy.

10.7 Second Malignancy

A *second malignancy* is one unrelated to the treatment of a prior malignancy (and is NOT a metastasis from the initial malignancy). Second malignancies require ONLY routine AE reporting unless otherwise specified.

11.0 STUDY CALENDAR

Data is to be submitted according to the Data submission timelines located on the PBTC-053 webpage.

11.1 Phase I and Surgical Study

	Screening	Pre-therapy for Phase I Patients	Pre-therapy for Phase I + Surgery Patients	Surgery	Post- Surgery	Course 1	Courses 2- Course 26	Completion/ Discontinuation Off Treatment
Screening Consent	X							
Informed Consent		X	X ^A					
CX-4945 drug administration			5-7 days pre surgery			Day 1-28, twice/day	Day 1-28, twice/day	
Surgery				X				
Medical history		X	X		X	Weekly	X	X
Physical exam		X	X		X	Weekly	X	X
Vital signs/weight		X	X		X	Weekly	X	X
Height		X	X		X	X	X	X
Performance status		X	X		X		X	X
Neurologic exam		X	X		X		X	X
<i>CBC</i> ^B WBC, Hgb, Hct, Platelets, ANC, ALC		X	X		X	Weekly	X ^B	X
<i>Serum Chemistry</i> ^B Sodium, Potassium, Bicarbonate, Chloride, Calcium, BUN, Creatinine, Glucose, Phosphorous, Magnesium, Albumin, Total Protein, SGPT(ALT), SGOT(AST), Total Bilirubin		X	X		X	Weekly	X ^B	X
Serum or Urine pregnancy test (for females of childbearing potential)		X ^C	X ^C		X ^C		X ^C	
CSF cytology (if clinically indicated) ^D		X	X				X	
25-OH Vitamin D level		X	X				Every 3 months	
12-lead EKG ^E		X	X		X	X ^E	X ^E	

	Screening	Pre-therapy for Phase I Patients	Pre-therapy for Phase I + Surgery Patients	Surgery	Post- Surgery	Course 1	Courses 2- Course 26	Completion/ Discontinuation Off Treatment
Imaging Assessments								
Brain MRI (standard) with diffusion		X	X	X ^F	X ^F		X ^F	X
Spinal MRI (if clinically indicated)		X	X				X ^G	X
Right knee MRI		X	X				X ^H	
Bone age x-ray ^I	X							
Patients with extraneural disease								
Computed Tomography scan (CT) or MRI ^I		X	X				X ^J	X
FDG PET ^K		X	X				X ^K	X
Bone Marrow ^L		X	X				X ^L	X
Correlative Studies	Refer to Section 5.1.2 for Sample Collection Schedule for all correlative studies							
A. Informed consent for Phase I + surgical study B. To be done more frequently if required to monitor toxicities C. Within 72 hours prior to starting the course D. As clinically indicated to follow disease at the discretion of the primary treating physician. It is recommended to follow at the same interval as required MRIs, if positive at the initiation of therapy. E. To be completed 2 hours (±1 hour) after the first dose of CX-4945 and prior to C2. To be completed in subsequent courses as clinically indicated following cardiology consult for QTc >480. F. MRI Brain intraoperatively or post-operatively within 4 weeks of starting Phase I/II treatment. MRI Brain while on study is to be done at the end of course 2, 4, 6, then every 3 months until off treatment, and at the end of treatment G. MRI Spine should be done at the same time points as the standard MRI brain, if clinically indicated. H. MRI Knee for Phase I patients on study should be done at the end of course 2, 4, 6, then every 3 months until progression or off treatment to monitor for growth plate abnormalities following treatment with CX-4945. I. To be performed only in patients ≤ 18 years within four (4) weeks prior to starting therapy. Bone age is to be repeated if growth plate abnormalities are seen on MRI of the knee. J. Extraneural disease sites should be imaged with MRI or CT as clinically indicated at baseline, the end of course 2, 4, 6, then every 3 months until off treatment, and at the end of treatment. Repeat tumor response evaluations should be done using the same modality as baseline assessments and address each site of disease affected. K. For patients with bone disease, obtain FDG PET scan at baseline, the end of course 2, 4, 6, then every 3 months until off treatment, and at the end of treatment. L. For patients with bone disease, perform bone marrow biopsy and aspirate at baseline. It is recommended to follow bone marrow disease at the same interval as imaging if positive at baseline, and as clinically indicated.								

11.2 Phase II and Surgical Study

	Screening	Pre-therapy for Phase II Patients	Pre-therapy for Phase II + Surgery Patients	Surgery	Post- Surgery	Course 1	Courses 2- Course 13	Courses 14-26	Completion/ Discontinuation Off Treatment
Screening Consent	X								
Informed Consent		X ^A	X ^A						
CX-4945 drug administration			5-7 days pre surgery			Day 1-28, twice/ day	Day 1-28, twice/ day	Day 1-28, twice/ day	
Surgery				X					
Medical history		X	X		X	Weekly	X	every 3 months	X
Physical exam		X	X		X	Weekly	X	every 3 months	X
Vital signs/weight		X	X		X	Weekly	X	every 3 months	X
Height		X	X		X	X	X	every 3 months	X
Performance status		X	X		X	X	X	every 3 months	X
Neurologic exam		X	X		X	X	X	every 3 months	X
CBC ^B WBC, HgB, Hct, Platelets, ANC, ALC		X	X		X	Weekly	X ^B	every 3 months ^B	X
Serum Chemistry ^B Sodium, Potassium, Bicarbonate, Chloride, Calcium, BUN, Creatinine, Glucose, Phosphorous, Magnesium, Albumin, Total Protein, SGPT(ALT), SGOT(AST), Total Bilirubin		X	X		X	Weekly	X ^B	every 3 months ^B	X
Serum or Urine pregnancy test (for females of childbearing potential)		X ^C	X ^C		X ^C	X	X ^C	X ^C	

	Screening	Pre-therapy for Phase II Patients	Pre-therapy for Phase II + Surgery Patients	Surgery	Post- Surgery	Course 1	Courses 2- Course 13	Courses 14-26	Completion/ Discontinuation Off Treatment
CSF cytology (if clinically indicated) ^D		X	X				X	X	
25-OH Vitamin D level		X	X				Every 3 months	Every 3 months	
12-lead EKG ^E		X	X		X	X ^E	X ^E		
Imaging Assessments									
Brain MRI (standard) with diffusion		X			X ^F		X ^F	X ^F	X
Spinal MRI (if clinically indicated)		X					X ^G	X ^G	X
Bone age x-ray ^H	X								
Patients with extraneural disease									
Computed Tomography (CT) or MRI ^I		X	X				X ^I	X ^I	X
FDG PET ^J		X	X				X ^J	X ^J	X
Bone Marrow ^K		X	X				X ^K	X ^K	X
Correlative Studies	Refer to Section 5.1.2 for Sample Collection Schedule for all correlative studies								
A. Informed consent for Phase II + surgical study									
B. To be done more frequently if required to monitor toxicities									
C. Within 72 hours prior to starting the course									
D. As clinically indicated to follow disease at the discretion of the primary treating physician. It is recommended to follow at the same interval as required MRIs, if positive at the initiation of therapy.									
E. To be completed 2 hours (±1 hour) after the first dose of CX-4945 and prior to C2. To be completed in subsequent courses as clinically indicated following cardiology consult for QTc >480.									
F. MRI Brain intraoperatively or post-operatively within 4 weeks of starting Phase I/II treatment. MRI Brain while on study is to be done at the end of course 2, 4, 6, 9, then every 3 courses until progression or off treatment, and at the end of treatment									
G. MRI Spine should be done at the same time points as the standard MRI brain, if clinically indicated.									
H. To be performed only in patients ≤ 18 years within four (4) weeks prior to starting therapy									
I. Extraneural disease sites should be imaged with MRI or CT as clinically indicated at baseline, the end of course 2, 4, 6, then every 3 months until off treatment, and at the end of treatment. Repeat imaging should be done using the same modality as baseline assessments and address each site of disease affected.									
J. For patients with bone disease, obtain FDG PET scan at baseline, the end of course 2, 4, 6, then every 3 months until off treatment, and at the end of treatment.									
K. For patients with bone disease, perform bone marrow biopsy and aspirate at baseline. It is recommended to follow bone marrow disease at the same interval as imaging if positive at baseline, and as clinically indicated.									

12.0 MEASUREMENT OF EFFECT

Although the clinical benefit of the CX-4945 drug has not yet been established, the intent of offering this treatment is to provide a possible therapeutic benefit, and thus the patient will be carefully monitored for tumor response and symptom relief in addition to safety and tolerability. Patients with measurable disease will be assessed by standard criteria. For the purposes of this study, patients should be re-evaluated every 8 weeks for the first 6 courses (Courses 2, 4, 6), then every 12 weeks (Courses 9, 12, 15, 18, 21, 24, 26) thereafter until time of progression or completion of treatment. In addition to a baseline scan, confirmatory scans will also be obtained 8 weeks following initial documentation of an objective response.

12.1 Antitumor Effect Definitions

- Evaluable for Toxicity

Patients who receive at least 1 dose of CX-4945 and are removed from treatment for toxicity during the dose-finding period (first 4 weeks of treatment) are evaluable for estimating the MTD.

Patients who receive approximately 85% ($\geq 24/28$ of required doses based on dosing schedule) of prescribed therapy during the dose-finding period but who progress prior to completing the course may be considered evaluable for estimating the MTD, as long as no additional anti-cancer therapy or supportive care that would confound the interpretation of any observed toxicity or side effect is given. Patients must have completed all of the clinical and laboratory monitoring requirements specified by the protocol up to the time of disease progression for them to be considered evaluable for MTD.

Patients who have completed all therapy during the dose-finding period but who failed to comply with all the specified clinical and laboratory monitoring requirements for the first course may be considered inevaluable for estimating the MTD and replaced.

Patients who receive less than 85% (miss more than 4 doses) of the protocol specified therapy and who go off treatment for reasons other than toxicity (e.g., dosing error, progressive disease, withdrawal of consent etc.) during the dose finding period will be considered inevaluable for estimating the MTD and will be replaced.

- Evaluable for Efficacy

All patients who receive at least 1 dose of the agent will be considered evaluable for efficacy analyses and will be assessed based on the criteria described in [Section 12.3](#).

- Evaluable for determination of intra-tumoral CX-4945 concentration on the surgical study

In order for patients to be considered evaluable, for the surgical component, samples from the surgical resection must be considered viable tumor and not necrotic material by the neurosurgeon and must meet the size requirements as specified in [Section 5.3.1.2](#). Please note patients who may not be evaluable for the PK study may still be evaluable for the Phase I expansion or Phase II studies should they proceed to those components of the study.

12.1.1 Disease Parameters

Sites should report tumor measurements in 3 dimensions in the database. In order to completely document the assessment of response, the measurements of the longest tumor dimension, and its perpendicular, of all target lesions upon which the assessments of tumor response are based should be explicitly noted in the radiology report for the baseline and all subsequent follow-up exams. Reports for the follow-up exams should reiterate the measurements obtained at baseline for each target lesion. Non-target lesions or newly occurring lesions should also be enumerated in these reports, and changes in non-target lesions should be described. The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up.

Tumor response criteria are determined by changes in size using the longest tumor dimension and its perpendicular. FLAIR, T2 or post contrast T1 weighted images may be used - whichever gives the best estimate of tumor size.

Since many tumors contain non-enhancing components (or, in some cases, the tumor may not enhance at all), both the enhancing and the non-enhancing components must be evaluated – on post contrast T1 weighted images and on FLAIR/T2 weighted images respectively. Increase in enhancement on T1 weighted images without accompanying increase in disease bulk on T2 or FLAIR images is not considered tumor progression. In return, enlarging areas of non-enhancing tumor (defined as mass effect/tissue thickening) are evidence of tumor progression. Conversely, decrease in enhancing tumor component without decrease in overall FLAIR/T2 extent may represent change in tumor permeability (commonly observed with antiangiogenic therapies) rather than represent tumor response.

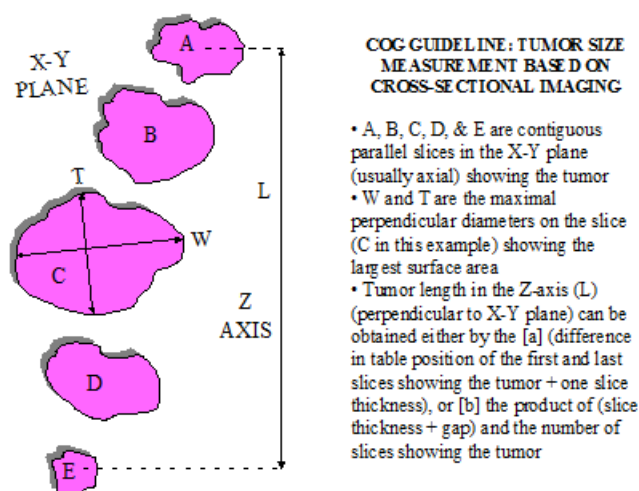
12.1.2 Method

The following section describes the methodology. (See Figure 8 below for illustration.)

- For MRI imaging (preferred), the longest measurement of the tumor (or width, W) should be determined. It can be measured from the axial plane or the plane in which the tumor is best seen or measured, provided the same plane is used in follow ups. Longest diameter of target lesion(s) should be selected in the axial plane only for CT.
- The measurement (transverse (T)) perpendicular to the width in the selected plane should be determined. NOTE: A measurable lesion should have a minimal transverse measurement that is at least twice the combined thickness of the image slice and the interslice gap. For example, with a 4 mm slice and a 0.4 mm gap, minimal measurable lesion diameter is 8.8 mm. Smaller lesions would not be measurable for study purpose.
- The cystic or necrotic components of a tumor are not considered in tumor measurements. Therefore, only the solid component of cystic/necrotic tumors should be measured. If cysts/necrosis composes the majority of the lesion, the lesion may not be “measurable”.
Options:
 - if the cyst/necrosis is eccentric, the W and T of the solid portion should be measured, the cyst/necrosis excluded from measurement

- if the cyst/necrosis is central but represents a small portion of the tumor (< 25%), disregard and measure the whole lesion
- if the cyst/necrosis is central but represents a large portion of the tumor, identify a solid aspect of the mass that can be reproducibly measured
- Leptomeningeal tumor spread is usually not a target lesion, and usually cannot be measured accurately. Presence and location of leptomeningeal tumor spread should be noted, and change in extent/thickness assessed on follow up studies.

Figure 8



12.1.3 Overall Response Assessment

The overall response assessment takes into account response in both target and non-target lesion, and the appearance of new lesions, where applicable, according to the criteria described in the table below. The overall response assessment is shown in the last column, and depends on the assessments of target, non-target, and new lesions in the preceding columns.

Overall Response Assessment

Target Lesions	New Lesions*	Overall Response
CR	No	CR
PR	No	PR
SD	No	SD
PD	Yes or No	PD
Any	Yes	PD
CR – Complete Response; PD – Progressive Disease; PR – Partial Response; IR – Incomplete Response; SD – Stable Disease *If CSF cytology becomes positive, it will be considered a new lesion and progressive disease.		

12.1.4 Selection of Target and Non-target Lesions

- For most CNS tumors, only one lesion/mass is present and therefore is considered a “target” for measurement/follow up to assess for tumor progression/response.
- If multiple measurable lesions are present, a minimum of the 2 largest lesions should be measured; a maximum of 5 should be selected as “target” lesions. Target lesions should be selected on the basis of size and suitability for accurate repeated measurements. All other lesions will be followed as non-target lesions.
- The lower size limit of the target lesion(s) should be at least twice the thickness of the slices showing the tumor to decrease the partial volume effect (e.g., 8 mm lesion for a 4 mm slice).

12.2 Tumor Response Criteria

12.2.1 Complete Response (CR)

Complete disappearance on MR/CT of all evaluable tumor and mass effect, on a stable or decreasing dose of corticosteroids (or receiving only adrenal replacement doses), accompanied by a stable or improving neurologic examination, and maintained for at least 8 weeks. If CSF was positive, it must be negative. If bone marrow was positive it must be free of tumor. Patients with FDG-PET avid bone disease at baseline must have no uptake upon reassessment.

12.2.2 Partial Response (PR)

Greater than or equal to 50% reduction in tumor size by product of the bi-dimensional measurements, as compared with the baseline measurements, on a stable or decreasing dose of corticosteroids, accompanied by a stable or improving neurologic examination, and maintained for at least 8 weeks. Patients with bone disease must have $\geq 50\%$ reduction in number of FDG-PET avid bone lesions. If bone marrow was positive, it must be free of tumor.

12.2.3 Stable Disease (SD)

Neurologic exam is at least stable and maintenance corticosteroid dose not increased, and imaging meets neither the criteria for PR nor the criteria for Progressive Disease. If this category is to be reported as of possible clinical benefit, Stable Disease status must be maintained for 4 courses.

12.2.4 Progressive Disease (PD)

Progressive Disease (PD): Progressive neurologic abnormalities or worsening neurologic status not explained by causes unrelated to tumor progression (e.g., anticonvulsant or corticosteroid toxicity wean, electrolyte disturbances, sepsis, hyperglycemia, etc.), OR a greater than 25% increase in the product of bi-dimensional measurements, taking as a reference the smallest disease measurement recorded since the start of protocol therapy, OR the appearance of a new tumor lesion. Development of new disease or progression/relapse in any established lesion(s), regardless of response in other lesions (e.g., when multiple lesions show opposite responses) will be considered progressive disease.

Increasing doses of corticosteroids required to maintain stable neurological status should be strongly considered as a sign of clinical progression unless in the context of recent wean or transient neurologic change.

12.2.5 Progression-free Survival

Interval of time between date of initiation of protocol treatment and minimum date of documentation of PD, second malignancy, death due to any cause, or date of last follow-up.

12.2.6 Duration of Response

- Duration of overall response: The duration of overall response is measured from the time measurement criteria are met for CR or PR (whichever is first recorded) until the first date that recurrent or progressive disease is objectively documented (taking as reference for progressive disease the smallest measurements recorded since the treatment started). The duration of overall CR is measured from the time measurement criteria are first met for CR until the first date that progressive disease is objectively documented.
- Duration of stable disease: Stable disease is measured from the start of the treatment until the criteria for progression are met, taking as reference the smallest measurements recorded since the treatment started, including the baseline measurements.

13.0 STUDY OVERSIGHT AND DATA REPORTING/ REGULATORY REQUIREMENTS

Adverse event lists, guidelines, and instructions for AE reporting can be found in [Section 10](#) (Adverse Events: List and Reporting Requirements).

13.1 Study Oversight

This protocol is monitored at several levels, as described in this section. The Protocol Principal Investigator is responsible for monitoring the conduct and progress of the clinical trial, including the ongoing review of accrual, patient-specific clinical and laboratory data, and routine and serious adverse events; reporting of expedited adverse events; and accumulation of reported adverse events from other trials testing the same drug(s). The Protocol Principal Investigator and statistician have access to the data at all times.

All Study Investigators at participating sites who register/enroll patients on a given protocol are responsible for timely submission of data via Medidata Rave and timely reporting of adverse events for that particular study. This includes timely review of data collected on the electronic CRFs submitted via Medidata Rave. All studies are also reviewed in accordance with the Pediatric Brain Tumor Consortium's (PBTC) data safety monitoring plan.

13.2 Data Reporting

Medidata Rave is a clinical data management system being used for data collection for this trial/study. Access to the trial in Rave is controlled through the CTEP-IAM system and role assignments.

Requirements to access Rave via iMedidata:

- Active CTEP registration with credentials necessary to access secure NCI/CTSU IT systems; and
- Assigned a Rave role on the LPO or PO roster at the enrolling site of: Rave CRA, Rave Read Only, Rave CRA (LabAdmin), Rave SLA, or Rave Investigator.

Rave role requirements:

- Rave CRA or Rave CRA (Lab Admin) role must hold a minimum of an, Associate Plus (AP) registration type;
- Rave Investigator role must be registered as a Non-Physician Investigator (NPIVR) or Investigator (IVR); and
- Rave Read or Rave RLA Only role must have at a minimum an Associate (A) registration type.

Refer to <https://ctep.cancer.gov/investigatorResources/default.htm> for registration types and documentation required.

This study has a Delegation of Tasks Log (DTL). Therefore, those requiring write access to Rave must also be assigned the appropriate Rave tasks on the DTL.

Upon initial site registration approval for the study in Regulatory application, all persons with Rave roles assigned on the appropriate roster will be sent a study invitation e-mail from iMedidata. No action will be required; each study invitation will be automatically accepted and study access in Rave will be automatically granted. Site staff will not be able to access the study in Rave until all required Medidata and study specific trainings are completed. Trainings will be in the form of electronic learnings (eLearnings) and can be accessed by clicking on the eLearning link in the *Tasks* pane located in the upper right corner of the iMedidata screen. If an eLearning is required for a study and has not yet been taken, the link to the eLearning will appear under the study name in the *Studies* pane located in the center of the iMedidata screen; once the successful completion of the eLearning has been recorded, access to the study in Rave will be granted, and a *Rave EDC* link will replace the eLearning link under the study name.

No action will be required by site staff (to activate their account) who have not previously activated their iMedidata/Rave account at the time of initial site registration approval for the study in the Regulatory application. Pending study invitations (previously sent but not accepted or declined by a site user) will be automatically accepted and study access in Rave will be automatically granted for the site user. Account activation instructions are located on the CTSU website in the *Data Management* section under the Data Management Help Topics > Rave resource materials (*Medidata Account Activation* and *Study Invitation*). Additional information on iMedidata/Rave is available on the CTSU members' website in the Data Management > Rave section or by contacting the CTSU Help Desk at 1-888-823-5923 or by e-mail at ctscontact@westat.com.

13.2.1 Method

This study will be monitored by the Clinical Data Update System (CDUS) Version 3.0. Cumulative protocol- and patient-specific CDUS data will be submitted electronically to CTEP on a quarterly basis by FTP burst of data. Reports are due January 31, April 30, July 31, and October 31. Instructions for submitting data using the CDUS can be found on the CTEP Web site (<http://ctep.cancer.gov/reporting/cdus.html>).

PBTC-053 is assigned to CDUS-Abbreviated reporting therefore no adverse event reporting (routine or expedited) is required to be reported via CDUS, but expedited adverse events are still required to be submitted via CTEP-AERS.

13.2.2 Responsibility for Data Submission

The OBDMC for the PBTC is responsible for compiling and submitting CDUS data to CTEP for all participants and for providing the data to the Principal Investigator for review.

13.3 Clinical Trial Agreement

A clinical trial agreement will be in place to document study operations between Senhwa Biosciences, Inc. and the PBTC. A Materials Transfer Agreement will be in place to document correlative study operations between the applicable labs and analyzing centers, and the PBTC.

13.4 Participant and Data Confidentiality

Participant confidentiality is strictly held in trust by the participating investigators, their staff and the sponsor(s) and their agents. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. Therefore,

the study protocol, documentation, data and all other information generated will be held in strict confidence. No information concerning the study and the data will be released to an unauthorized third party without the prior written approval of the Pediatric Brain Tumor Consortium (PBTC).

The PBTC protocol coordinators, other authorized representatives of the sponsor, regulatory representatives, PBTC auditors, representatives of the IRB or the pharmaceutical collaborator supplying study product may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

Source documents which are the original records of clinical findings, observations or activities in a clinical trial are to be maintained at each participating site. Sites must upload all source documentation which supports the eligibility, DLT or dose finding and evaluability of the participant to the PBTC via the RAVE database. In the event the patient experiences unexpected events, additional source documentation may be requested to complete the event review. These documents may include but are not limited to, hospital records, clinical and office charts, laboratory notes, memoranda and radiographic images.

Study participant study related data, which is for purposes of statistical analysis and scientific reporting will be transmitted to the Pediatric Brain Tumor Consortium electronically via the RAVE database. This will not include the participant's contact or identifying information. Rather, research participants and their research data will be identified by a unique study identification number assigned at the time of screening or registration. The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by local IRB and institutional regulations.

The study data entry and study management systems used by clinical sites and by the PBTC will be secured and password protected. At the end of the study, all study data is maintained on a secure server.

After the study is completed, the data collected will be maintained on a server and may be used by other investigators, including those outside the study. With the participant's approval and as approved by local IRBs, biological samples labeled only with the participant's protocol specific identification number will be stored at the PBTC Central Review and Biorepository and could be made available to other investigators for future unspecified research. Investigators conducting future studies will not have access to the key for stored data collected while the participant is on study. Clinical data will be de-identified before it is shared with other investigators.

If the participant agrees to submit a repository sample, those samples contain genetic information that may be used for research related to brain tumors and their treatment. They may also be used to develop tests/assays to improve diagnosis and treatment of these diseases in the future. Genetic research may consist of the analysis of one or more genes or the analysis of genetic markers throughout the genome.

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APPENDIX A Performance Status Criteria

MODIFIED LANSKY SCORE (Score as 0 - 100)

A. Normal Range

- 100 = Fully active
- 90 = Minor restrictions in physically strenuous play
- 80 = Restricted in strenuous play, tires more easily, otherwise active

B. Mild to moderate restriction

- 70 = Both greater restrictions of and less time spent in active play
- 60 = Ambulatory up to 50% of time, limited active play with assistance/supervision
- 50 = Considerable assistance required for any active play; fully able to engage in quiet play

C. Moderate to severe restriction

- 40 = Able to initiate quiet activities
- 30 = Needs considerable assistance for quiet activity
- 20 = Limited to very passive activity initiated by others e.g., TV)
- 10 = Completely disabled, not even passive play
- 0 = Unresponsive, coma

KARNOFSKY SCALE

- 100 = Normal; no complaints
- 90 = Able to carry on normal activities; minor signs or symptoms of disease
- 80 = Normal activity with effort
- 70 = Cares for self. Unable to carry on normal activity or to do active work
- 60 = Requires occasional assistance but able to care for most of his/her needs
- 50 = Requires considerable assistance and frequent medical care
- 40 = Disabled; requires special care and assistance
- 30 = Severely disabled; hospitalization indicated though death not imminent
- 20 = Very sick. Hospitalization necessary. Active support treatment necessary.
- 10 = Moribund
- 0 = Dead

APPENDIX B Patient Drug Information Handout and Wallet Card

Information for Patients, Their Caregivers, and Non-Study Healthcare Team on Possible Interactions with Other Drugs and Herbal Supplements

The patient _____ is enrolled on a clinical trial using the experimental study drug, **CX-4945**. This form is addressed to the patient, but includes important information for others who care for this patient.

To the patient: Take this paper with you to your medical appointments and keep the attached information card in your wallet.

CX-4945 may interact with other drugs that are processed by your liver and can cause unexpected side effects. For this reason, it is very important to tell your study doctors of any medicines you are taking before you enroll onto this clinical trial. It is also very important to tell your doctors if you stop taking any regular medicines, or if you start taking a new medicine while you take part in this study. When you talk about your current medications with your doctors, include medicine you buy without a prescription (over-the-counter remedy), or any herbal supplements such as St. John's Wort. It is helpful to bring your medication bottles or an updated medication list with you.

Many health care providers can write prescriptions. You must tell all of your health care providers (doctors, physician assistants, nurse practitioners, pharmacists) you are taking part in a clinical trial.

These are the things that you and they need to know:

CX-4945 should be used very carefully with other medicines. Before you enroll onto the clinical trial, your study doctor will work with your regular health care providers to review any medicines and herbal supplements which you are taking.

- Please be very careful! Over-the-counter drugs (including herbal supplements) may contain ingredients that could interact with your study drug. Speak to your doctors or pharmacist to determine if there could be any side effects.
- Warfarin is prohibited.
- Your regular health care provider should check a frequently updated medical reference or call your study doctor before prescribing any new medicine or discontinuing any medicine. Your study doctor's name is _____ and he or she can be contacted at _____.

STUDY DRUG INFORMATION WALLET CARD

You are enrolled on a clinical trial using the experimental study drug **CX-4945**. This clinical trial is sponsored by the NCI. **CX-4945 may interact with other drugs.** Because of this, it is very important to:

- Tell your doctors if you stop taking any medicines or if you start taking any new medicines.
- Tell all of your health care providers (doctors, physician assistants, nurse practitioners, or pharmacists) that you are taking part in a clinical trial.
- Check with your doctor or pharmacist whenever you need to use an over-the-counter medicine or herbal supplement.

CX-4945 must be used very carefully with other medicines.

- Before you enroll onto the clinical trial, your study doctor will work with your regular health care providers to review any medicines and herbal supplements which you are taking
- Warfarin is prohibited.
- Before prescribing new medicines, your regular health care providers should go to a frequently-updated medical reference for a list of drugs to avoid, or contact your study doctor.
- Your study doctor's name is _____ and can be contacted at _____.

APPENDIX C Dosing Tables

PHASE I:

Dose Level 0: 400 mg/m² BID

Minimum BSA must be $\geq 0.84\text{m}^2$ and the maximum BSA is 2.25m^2

CX-4945 Dose Level 400 mg/m ² BID	BID Dose (mg)	BSA Range (m ²)		
		low	high	Number of 200 mg Capsules
	400	0.84	1.25	2
	600	1.26	1.74	3
	800	1.75	2.25	4

Dose Level 1: 600 mg/ m² BID

Minimum BSA must be $\geq 0.60\text{m}^2$ and the maximum BSA is 2.00m^2

CX-4945 Dose Level 600 mg/m ² BID	BID Dose (mg)	BSA Range (m ²)		
		low	high	Number of 200 mg Capsules
	400	0.60	0.83	2
	600	0.84	1.16	3
	800	1.17	1.50	4
	1000	1.51	2.00	5

Dose Level 2: 800 mg/m² BID

Minimum BSA must be $\geq 0.63\text{m}^2$ and the maximum BSA is 2.00m^2

CX-4945 Dose Level 800 mg/m ² BID	BID Dose (mg)	BSA Range (m ²)		
		low	high	Number of 200 mg Capsules
	600	0.63	0.83	3
	800	0.84	1.12	4
	1000	1.13	1.37	5
	1200	1.38	1.62	6
	1400	1.63	2.00	7

PHASE II:

Dose Level 0:

400 mg/m² BID - For Patients ≤18 years **AND** BSA ≤1.74 m²

Minimum BSA must be ≥0.60m², BSAs in the range of 0.75-0.83m², inclusive, are excluded at this dose level

CX-4945 Dose Level 400 mg/m ² BID		BSA Range (m ²)		
		low	high	Number of 200 mg Capsules
	BID Dose (mg)			
	200	0.60	0.74	1
	400	0.84	1.25	2
	600	1.26	1.74	3
Patients older than 18 years OR with BSA >1.74 m ² will receive a flat dose of 800mg.				

Dose Level 1:

600 mg/ m² BID - For Patients ≤18 years **AND** BSA ≤1.50

Minimum BSA must be ≥0.60m²

CX-4945 Dose Level 600 mg/m ² BID		BSA Range (m ²)		
		low	high	Number of 200 mg Capsules
	BID Dose (mg)			
	400	0.60	0.83	2
	600	0.84	1.16	3
	800	1.17	1.50	4
Patients older than 18 years OR with BSA >1.50 m ² will receive a flat dose of 1000mg.				

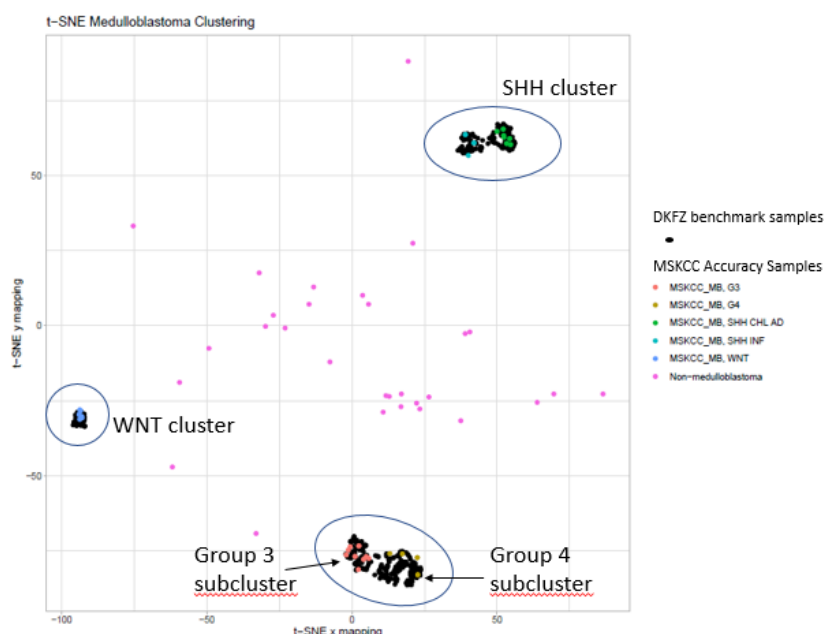
APPENDIX D1 Medulloblastoma (MB) Subgrouping Validation – MSKCC

Narrative Summary-MB Subgrouping at MSKCC	The MSKCC clinical assay for medulloblastomas subgroup utilizes random forest based brain tumor classification algorithm developed by The University Hospital Heidelberg, the German Cancer Research Center (DKFZ), and the German Consortium for Translational Cancer Research (DKTK) and is based on the strong association between genome-wide methylation profiles and clinically important central nervous system neoplasms as defined by the 2016 World Health Organization Classification of Tumours of the central nervous system (see PMID 29539639). In brief, the classifier uses the 10,000 most predictive CpG sites on the array platform to build 10,000 binary decision trees that each ‘vote’ for a brain tumor classification (also known as methylation class). The classifier was trained on reference cohort of 2,801 CNS tumors across almost all WHO categories. The final output is a list of calibrated scores (one score for each methylation class) that sum to 1.0. In general, the more ‘votes’ for the particular classification, the higher the score of the classification. The performance of the classifier was subsequently evaluated by comparison of the predicted methylation class to the diagnosis obtained by standard histopathologic and molecular analysis (blinded to methylation results) on a prospective cohort of 1,104 tumor samples (71% adult, 29% pediatric). Overall, across 91 tumor and non-tumor classes, the classifier demonstrated 88% accuracy. The validation was also performed at multiple institutions to ensure reproducibility. For medulloblastomas, specifically, the training/reference set comprised a high-quality cohort of 390 genetically-defined medulloblastomas (39 WNT, 136 SHH, 77 Group 3, and 138 Group 4). By methylation clustering these four consensus groups are divided into one methylation class (WNT) and four methylation subclasses (two SHH subclasses, Group 3 subclass, and Group 4 subclass). The two SHH subclasses are associated with distinct clinical features (a child/adult subtype and infant subtype) that together comprise the SHH methylation class family that corresponds to the SHH subgroup. The Group 3 and Group 4 subclasses constitute the Group 3 and 4 subgroups, respectively. Due to their epigenetic overlap, they are also used to define a Group 3/4 methylation class family. The prospective validation of the classifier included 123 genetically-defined medulloblastomas (51 SHH, 8 WNT, 18 Group 3, 42 Group 4, and 4 unclassifiable/NOS). Of the 119 genetically classifiable medulloblastomas, the classifier correctly subgrouped every sample (100% accuracy; see PMID 29539639, supplementary data). The performance characteristics of this methylation-based subgrouping technique was further assessed in a validation study performed at MSKCC using an internal cohort of medulloblastomas. Accuracy/consistency, reproducibility, analytical sensitivity, and limit of detection studies were performed and are summarized below. Additional details of this validation study may be provided upon request. To carry out the accuracy/consistency study, a total of 30 medulloblastomas and 30 non-medulloblastomas were subjected to methylation-based subgrouping. The 30 non-medulloblastoma brain tumors, encompassing a range of WHO categories (gliomas, meningiomas, melanomas, pituitary tumors, pineal tumors, etc.), were used to assess specificity. As methylation-based subgrouping is considered a gold-standard method for medulloblastomas, the methylation-based subgroup was compared to orthogonal testing correlates (histologic and molecular) of the four subgroups to ensure results are consistent with the assigned methylation subgroup. Additionally, all samples were clustered with the
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benchmark reference cohort of 390 medulloblastomas (39 WNT, 84 SHH CHL AD, 52 SHH INF, 77 Group 3, and 138 Group 4) obtained from the DKFZ to visualize their epigenetic relationship to the four subgroups.

Next generation sequencing and in-house immunohistochemistry were used as orthogonal testing methods. In cases where these were not available, copy number profiles were obtained from the methylation array data to assess for copy number correlates of medulloblastoma subgroups.

All 30 medulloblastomas were assigned a medulloblastoma methylation class or subclass and all 30 non-medulloblastomas were assigned a non-medulloblastoma methylation class. Twenty-seven (27/30=90%) had a methylation class or methylation class family score above 0.8, indicating a very confident match. The three remaining cases had lower methylation class family scores (0.65, 0.70, and 0.73), indicating lower but still confident matches based on previously described performance characteristics of the classifier (see PMID 29967940). The lowest subclass score was 0.51 with a corresponding MCF score of 0.70, indicating a confident match and a medulloblastoma that shares epigenetic overlap between Group 3 and 4, a common phenomenon for Group 3 and 4 subgroups. Two showed orthogonal testing that was consistent with two different medulloblastoma subgroups (NGS detection of WNT associated mutations and SHH associated mutations.) This is a well-recognized phenomenon and evidence suggest that these are WNT subgroup tumors that acquire SHH mutations. Prior studies have shown that these tumors with dual WNT and SHH mutations cluster with the WNT methylation-based subgroup, consistent with our findings (see PMID 29435664). By clustering with the benchmark reference cohort, all medulloblastoma samples tightly localized to the reference clusters and non-medulloblastomas localize far away from clusters (see figure below).



A precision study was performed to assess the intra-assay variation of methylation-based medulloblastoma subgrouping for replicates within the same experimental run. Three

	medulloblastomas and three non-medulloblastomas (glioblastomas) were subgrouped in triplicate in the same run. A reproducibility study was performed to assess the inter-assay variation of methylation-based subgrouping for replicates between different experimental runs. Three medulloblastomas and three non-medulloblastomas (glioblastomas) were subgrouped across three different runs. Subgrouping showed excellent precision and reproducibility. All samples (both medulloblastomas and non-medulloblastomas) were assigned the same methylation class/subclass and methylation class family across and within all experiment runs with minimal score variation. An analytical sensitivity study of the assay for methylation based medulloblastoma subgrouping was performed via a serial dilution experiment using a WNT subgroup medulloblastoma diluted into a cerebellar sample that classified with high calibrated score to normal cerebellar hemisphere. In addition to the undiluted WNT and cerebellar samples, we made 4 serial dilutions (50%, 25%, 12.5%, 6.25%) of the WNT sample into the cerebellar sample. Classification accuracy is strong (scores > 0.94) for samples greater than or equal to 50%. At 25% and below, samples classified to normal cerebellum. This analytical sensitivity level is satisfactory for this assay as medulloblastomas are almost always highly cellular neoplasms with > 50% tumor cells. A limit of detection study was performed to determine the minimum amount of DNA required for accurate medulloblastoma subgrouping. A WNT-subgroup medulloblastoma was run at various DNA amounts (250ng to 25ng). The data show that reliable subgrouping is possible down to 25ng of input sample.
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APPENDIX D2 Medulloblastoma (MB) Subgrouping Validation – NCI

Acronyms and Abbreviations	ACMG - American College of Medical Genetics BCD - Bisulfite Converted DNA CAP - College of American Pathologists CLIA - Clinical Laboratory Improvement Amendments Of 1988 CMS - Center for Medicare & Medicaid Services CPS - Chromosome Pathology Section CRIS - Clinical Research Information System DLBCL - Diffuse Large B-cell Lymphomas DNA - Deoxyribonucleic Acid DNase - Deoxyribonuclease DI - Deionizer water FDA - U.S. Food and Drug Administration FF - Fresh Frozen FFPE - Formalin Fixed Paraffin Embedded GBM - Glioblastoma multiforme HHS - Department of Health and Human Services LIS - Laboratory Information System MGMT O6-methylguanidine-DNA-methyltransferase MRN - Medical Record Number NIST - National Institute of Standards and Technology ON - Over Night PT - Proficiency Testing PXA - Pleomorphic Xanthoastrocytoma QA - Quality Assessment QAP - Quality Assessment Plan QC - Quality Control QM - Quality Management RT - Room Temperature RNA - Ribonucleic Acid RNase - Ribonuclease TAT - Turn-around time TE - Tris-EDTA buffer WGA - Whole-Genome Amplification
Clinical Indication for Test	Accurate pathological diagnosis is critical for optimal management of patient with cancer. For approximately 100 known tumors types of central nervous system (CNS), standardization of the diagnostic process has been shown to be particularly challenging – with substantial inter-observer variability in the histopathological diagnosis of many tumor types. These tumors are clinically and biologically diverse, encompassing a wide spectrum from benign neoplasms, which can frequently be cured by surgery alone (for example, pilocytic astrocytoma), to highly malignant tumors that response poorly to any therapy (for example, glioblastoma). To address these issues, molecular grouping has been introduced into update of the WHO classification, but only for selected entities, such as medulloblastoma. Furthermore, several single-gene tests entities such as DNA methylation analysis (for example, MGMT promoter methylations status), fluorescence in situ hybridization (FISH, for example 1p / 19q codeletion, EGFR, MYC, MYCN, PDGFRA, 19q13.42) or immunohistochemistry (for example, CTNNB1 and LN28A) that are required to cover most important differential diagnoses have been shown to be difficult to standardize. Such

	diagnostic discordance and uncertainty may confound decision-making in clinical practice as well as the interpretation and validity of clinical trial results. DNA methylation is the most well characterized epigenetic mark in humans. It is defined as addition of a methyl C(CH₃) group to DNA and in mammalian cells occurs primarily at the cytosine of cytosine-guanidine dinucleotide (CpG). DNA methylation can modify the function of the regulatory elements and gene expression and is therefore integral to normal human development and biological functioning. Perturbation to normal DNA methylation patterns can lead to dysregulation of cellular processes and linked to diseases. Widespread aberrations in DNA methylation are well-established hallmarks on many cancers [1, 2] and growing body of literature shows a role for DNA methylation in the etiology of other human diseases including chronic kidney disease [3], type 2 diabetes [4], and neuropsychiatric disease [5]. DNA methylation-based classification of tumors using comprehensive machine-learning approach was recently developed by international team led by German investigators [6]. This team developed a DNA methylation-based classification scheme for classifying central nervous system (CNS) tumors. It has been convincingly shown that DNA methylation profiling is highly robust and reproducible even from small samples and poor-quality material [7], and such profiles have been widely used to sub-classify CNS tumors that were previously considered homogeneous diseases [5, 8-12]. The use of DNA methylation signatures in combination with histology and molecular tumor classification will improve diagnostic accuracy not only in neuropathology but will serve as a blueprint in other fields of tumor pathology. Classification of CNS tumors based on DNA methylation is useful in providing precision diagnostic information and guiding treatment for patients with tumor of CNS as well as some other tumor types.
Assay Description, Principle, and Validity	Specimen source Formalin fixed paraffin embedded (FFPE) samples represent majority (>90%) of all archived solid tumor samples stored in hospitals and tissue banks across the globe [13]. The genetic profiling of FFPE samples provides valuable clinical information for diagnosis, prognosis and prediction of treatment outcomes. Unfortunately, nucleic acids and other macromolecules suffer considerable deterioration in quantity and quality as results of routine FFPE process [14], complicating any downstream molecular assay. DNA isolated from FFPE are usually characterized by short fragment length, DNA-protein cross-links and base damage [15]. Another issue is the limited amount of DNA that can be extracted from such specimen. Such inherent features of DNA extracted from FFPE tissue can lead to assay failure, errors in amplification and sequencing, and subsequent misinterpretation of results. To overcome these problems, recently different methods for DNA restoration procedures consisting of DNA repair and ligation processes, were successfully developed. These methods produce adequate results for FFPE samples using the Illumina arrays and were validated in comparison with matched fresh frozen (FF) samples [16-19]. The Infinium FFPE DNA Restore Kit provides a simple workflow to repair damaged DNA, achieving high-quality data in Infinium assays. Thus, fast and accurate DNA methylation analysis methods are imperative for discovering the underlying role that methylation plays in the course of normal vs. diseased development. Epigenomic studies using FFPE tissues shall be implemented in clinical practice to aid fully characterize and classify tumors and to help with identification of novel biomarkers and developing targeted therapies.

Source of the array

The Infinium Methylation Beadchips (Illumina, Inc., San Diego, CA) provide exceptional coverage of CpG islands, RefSeq genes, ENCODE open chromatin, ENCODE transcription factor binding sites, and FANTOM5 enhancers (Figure 1). Infinium HD technology enables content selection independent of bias-associated limitations often associated with methylated DNA capture methods. With the Infinium MethylationEPIC Beadchip Kit, one can interrogate over 850,000 methylation sites quantitatively across the genome at single-nucleotide resolution. Multiple samples, including FFPE, can be analyzed in parallel to deliver high-throughput power while minimizing the cost per sample.

The Infinium Methylation Beadchips targets regions identified by the ENCODE project as potential enhancers. ENCODE tracks for open chromatin, FAIRE assays, and transcription factor binding sites were overlaid to identify the most dynamic regions of the genome for content. Finally, the array targets enhancers identified by the FANTOM5 project across tissue types. The result is a pan-enhancer and coding region view of the methylome that can be used for epigenome-wide association studies on various human tissues and includes the following content categories requested by methylation experts:

- 1. 865,918 total probes (CpG, CNG, and SNP)
- 2. 863,904 (99.7%) are CpG probes and 2,932 (0.3%) CNG targets
- 3. 636 control probes and 59 SNP probes for sample matching
- 4. CpG sites in and outside the CpG islands
- 5. Non-CpG methylated sites identified in human stem cells (CHH sites)
- 6. Differentially methylated sites identified in tumor versus normal (multiple forms of cancer) and across several tissue types
- 7. FANTOM5 enhancers
- 8. ENCODE open chromatin and enhancers
- 9. DNase hypersensitivity sites
- 10. miRNA promoter regions
- 11. 54% in promoters, 30% gene bodies, 16% in intergenic regions.

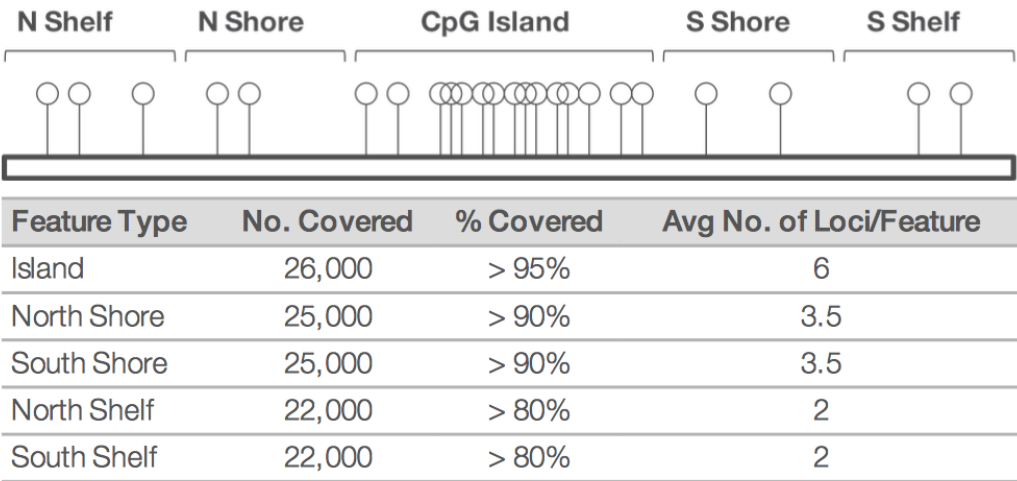


Figure 1. The Infinium MethylationEPIC beadchip coverage throughout gene regions. The Infinium MethylationEPIC Beadchip offers broad coverage across gene regions, CpG islands CpG island regions, shelves, and shores for a comprehensive view of the methylation state.

	Streamlined Workflow The Infinium MethylationEPIC Beadchip follows a user-friendly, streamlined workflow that does not require PCR. Its low sample input requirement (as low as 250 ng), enables analysis of valuable samples derived from limited DNA sources. Infinium MethylationEPIC protocol requires bisulfite DNA conversion, followed by FFPE restoration, amplification and hybridization. All procedures are performed using kits which contain all required reagents for performing complete workflow for methylation analyses. The data analysis workflow includes Illumina GenomeStudio which is an integrated data analysis software platform. GenomeStudio provides a common environment for quality control and analysis of the data obtained from Infinium microarray platform. The data then streamed into CNS Classifier which provides comprehensive machine-learned classification of tumor based on DNA methylation profile. Such approach was demonstrated to be a valuable asset in clinical practice. In particular, the high level of standardization has great promise to reduce the substantial inter-observer variability observed in current CNS tumor diagnostics. In addition, in contrast to traditional pathology, where there is a pressure to assign all tumor to a described entity even for atypical or challenging cases, the objective measure provided by Classifier allows for a ‘no match’ to a defined class. Such information can also be of substantial value to highlight that a tumor is not a typical example of a given differential diagnosis, and may belong to a rarer, currently undefined class.
Quality Control	Analytical Validation Due to a large number of CpG sites that can be simultaneously assessed using high-density bead array validation with well-characterized, positive genomic DNA controls for all possible methylated or unmethylated CpG states may not be possible. For some loci highly-methylated and hypo-methylated DNA controls can be obtained from commercial sources (Millipore, Epigentek, Qiagen and others) in form of isolated genomic DNA. When genomic control DNA is not available, it is possible to use synthetic controls produced by enzymatic procedures. The identity of such synthetic controls should be also verified by orthogonal methods such as, for instance, bisulfite conversion followed by DNA sequencing prior to use in microarray validation. Internal Control Analytes All Infinium Beadchips are equipped with a set of internal control probes designed to support quality control of the assay's stringent performance criteria and to demonstrate its robustness. High quality samples generally yield unambiguous genotype calls with average call rates of >99%. Built-in Infinium controls help to identify samples for which data characteristics are significantly different and may need to be excluded as outliers from further analysis. Also, Infinium controls facilitate troubleshooting by linking possible root causes to specific steps in the assay protocol. As intensity levels may be different for any given Infinium project, Infinium controls are not designed to perform quality control based on specific thresholds. Instead, Infinium controls are evaluated based on relative intensities. The Infinium assay controls are comprised of sample-independent controls and sample-dependent controls. <i>Sample-independent controls</i> are used to evaluate beadchip and reagent performance, efficiency of hybridization, and the staining process. They include Staining Controls, Extension Controls, Target Removal Controls, Hybridization Controls, and the Restoration Control. The Restoration Control is only informative if following the Infinium Formalin-Fixed Paraffin-Embedded (FFPE) workflow.

Sample-dependent controls are used to evaluate sample quality and performance. They include Bisulfite Conversion Controls I and II, Specificity Controls I and II, Non-Polymorphic and Negative Controls. Sample-dependent control probes specifically target human DNA and are not informative when working with non-human beadchip products.

Table 1. Infinium methylation controls.

Purpose	Name	Color channel	Expected Intensity
Staining	DNP (High)	Red	High
Staining	DNP (Bgnd)	Red	Background
Staining	Biotin (High)	Green	High
Staining	Biotin (Bgnd)	Green	Background
Extension	Extension (A), (T)	Red	High
Extension	Extension (C), (G)	Green	High
Hybridization	Hyb (Low)	Green	Low
Hybridization	Hyb (Med)	Green	Medium
Hybridization	Hyb (High)	Green	High
Target Removal	Target removal 1, 2	Green	Low
Bisulfite Conversion I	BC Conversion I C1, C2, C3	Green	High
Bisulfite Conversion I	BC Conversion I U1, U2, U3	Green	Background
Bisulfite Conversion I	BC Conversion I C4, C5, C6	Red	High
Bisulfite Conversion I	BC Conversion I U4, U5, U6	Red	Background
Bisulfite Conversion II	BC Conversion II 1, 2, 3, 4	Red	High
Specificity I	GT mismatch 1, 2,3, (PM)	Green	High
Specificity I	GT mismatch 1, 2,3, (MM)	Green	Background
Specificity II	GT mismatch 1, 2,3	Red	High
Non-polymorphic	NP (A), (T)	Red	High
Non-polymorphic	NP (C), (G)	Green	High
Negative	Average*	Red/Green	Background
Negative	StDev**	Red/Green	Background

*Average intensity of 600 negative control probes

**Standard deviation of intensities of 600 negative control probes

Bioinformatic Analysis Pipeline

Illumina GenomeStudio (Illumina, Inc., San Diego, CA) is an integrated data analysis software platform, which provides a common environment for analyzing data obtained from microarray and sequencing technologies. Within this common environment, or framework, different GenomeStudio software modules allows to perform application-specific analyses. The GenomeStudio Methylation Module is an application for extracting genome-wide DNA methylation information from data files collected from systems such as the Illumina iScan reader. Experiment performance is based on built-in controls that accompany each experiment. Data analysis features of the Methylation Module include:

1. Assay-specific Controls Dashboard
2. Differential methylation analysis
3. Choice of assay analysis within a single application

	4. Data tables for information management and manipulation 5. Plotting and graphing tools 6. Whole-genome display of sample data in the IGV (Illumina Genome Viewer) 7. Data visualization in the ICB (Illumina Chromosome Browser) 8. Data normalization 9. Custom report file formats <i>Methylation Classifier</i> is a comprehensive machine-learning approach for tumor classification that was developed from central nervous system (CNS) tumors using information from more than 2,801 reference samples and 91 methylation classes. The algorithm uses DNA methylation profile to assign a diagnostic classification to CNS tumors across all entities and age groups. Its accuracy and value in clinical decision-making was demonstrated in routine diagnostic setting [6]. Proficiency Testing Since the College of American Pathologist (CAP) does not offer proficiency testing surveys for methylation microarray testing, our laboratory will use an alternative assessment of proficiency for methylation testing by pyrosequencing and / or exchanging samples with certified laboratory performing similar testing.
Pilot Study	The purpose of pilot the study is assessment of Infinium methylation assay operating characteristics such as trueness, repeatability, linearity, etc. One method of assessing these parameters is to divide DNA sample into multiple aliquots and hybridize this same DNA sample to multiple beadchips, then perform a variance component analysis. Alternatively, a DNA sample may be split with another laboratory that performs the same test. Significant CpG loci could be validated using pyrosequencing or another more traditional sequencing assay. We started this study with testing methylation of DNA samples from healthy individuals and well characterized in the literature cell lines. These experiments allowed as to get familiar with the protocol, instrumentation, data generation and analysis. Next, we assessed the accuracy of the assay by testing analytical sensitivity, specificity and reproducibility using normal and tumor DNA samples. Pre- Analytic Phase Supplementary info: the experimental procedures are described in the DNA Methylation-based Tumor Classification Standard Operating Procedure manual. Matrix stability: the testing is performed using DNA from FFPE tissue sections isolated and purified by Molecular Diagnostic laboratory, which is part of the Laboratory of Pathology. DNA is dissolved in low TE buffer and stored at +4oC for several months. Long term storage of the DNA sample is carried out at -20oC freezer. Quantitation of DNA were performed by using Qubit assay (Thermo Fisher Scientific, Waltham, MA). Analyte storage: according to manufacturer recommendations, the original beadchips are stored at +4oC refrigerator until expiration date, hybridized beadchips are stored at room temperature in light protected space and discarded after 3 months. Other Infinium Methylation kit components are stored according to manufacturer's recommendations. Specimen handling requirements: see DNA Methylation-based Tumor Classification Standard Operating Procedure manual.

	Analytic Phase Experimental procedure: To confirm the assay specificity i.e. correct hybridization and detection of the methylation status for regions to which beadchip probes are designed, we evaluated probes specificity using DNA specimen isolated from peripheral blood of eight healthy donors. For each donor genomic DNA were processed according to Infinium protocol: genomic DNA were bisulfite converted, amplified, fragmented, hybridized to MethylationEPIC beadchips and stained. Then beadchips were scanned on iScan reader and signal intensities for each specimen were recorded. Scanning of the beadchips. Infinium Beadchips are scanned on iScan reader (Illumina, Inc., San Diego, CA) in automatic mode. Each beadchip consist of eight hybridization areas for simultaneous analysis if eight DNA specimen. The unique barcode is printed on each beadchip in the label area and is read by iScan instrument. The signal intensity (idat-) files representing two, red and green, color channels are generated for each individual sample. The system creates a folder on a network drive using the beadchip barcode as a name and saves idat-, and system files in that folder for further analysis by GenomeStudio and by tumor Classifier. Post-Analytic Phase Bioinformatic Analysis. A Methylation Module in the GenomeStudio software enables to obtain and analyze methylation beta-values, signal intensities, and p-value measurements for sample and control probes. Analysis of the built-in Infinium controls help to identify samples for which data characteristics are significantly different and may need to be excluded as outliers from analysis. The data are easily exported into Excel-compatible spreadsheets for further analyses. GenomeStudio also has the ability to perform unsupervised two-dimensional clustering and generate heat maps to determine sample- and gene-methylation relationships. Tumor Classier is a software code which is run on NIH secure network and provides streamlined platform for data upload, automation normalization, QC analysis, tumor classification and report generation. Obtained report incorporates tumor classification with predictive score, DNA copy-number profile (CNV profile), and O6-methylguanidine-DNA-methyltransferase (MGMT) promoter methylation status [6]. System Controls. Infinium MethylationEPIC Beadchips are equipped with a set of internal control probes designed to support quality control of the assay's stringent performance criteria and to demonstrate its robustness. Infinium controls are not designed to perform quality control based on specific thresholds. Instead, Infinium controls are evaluated based on relative intensities. The Infinium assay controls are comprised of sample-independent controls and sample-dependent controls. At first step to check the quality of the data, we tested whether the DNA samples passes the quality controls. We evaluated the sample-dependent and sample-independent controls. All specimen had acceptable signal intensity levels for all controls and passed quality assessment. Reproducibility of the assay was accessed by hybridization of technical replicates of the DNA from eight specimens to the same and different beadchip arrays. We found a high correlation between β-values for seven out of eight samples of technical replicates (Table 2 and Figure 2). Specimen Q060 demonstrated lower R-squared coefficient compared to rest of the samples. Detailed analysis of this sample revealed lower quality of DNA, probably due to degradation, which affected bisulfite conversion and following steps of the procedure.
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Table 2. Reproducibility analysis using replicate samples*.

Replicates	M533	Q060	Q856	Q992	T215	T213	S462	T302
M533	0.97	0.82	0.83	0.83	0.83	0.78	0.87	0.86
Q060	0.76	0.87	0.84	0.88	0.87	0.84	0.87	0.80
Q856	0.80	0.83	0.99	0.93	0.90	0.85	0.91	0.86
Q992	0.79	0.86	0.93	0.99	0.94	0.89	0.93	0.84
T215	0.80	0.86	0.90	0.94	0.99	0.88	0.93	0.86
T213	0.75	0.81	0.85	0.89	0.88	0.99	0.88	0.79
S462	0.84	0.87	0.90	0.93	0.93	0.88	0.98	0.87
T302	0.84	0.81	0.88	0.87	0.88	0.81	0.89	0.99

*R-squared, R^2 , is calculated and shown for comparison of technical replicated

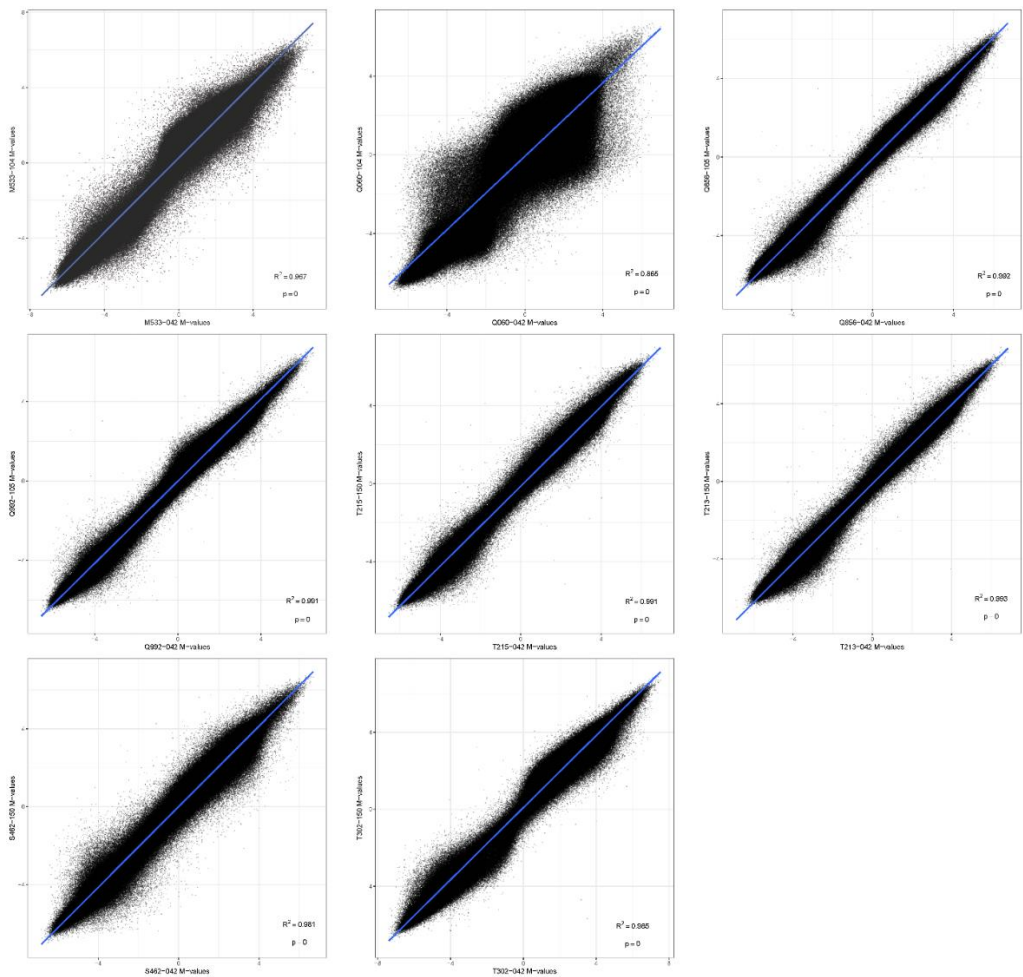


Figure 2. Reproducibility of methylation assay. Scatter plots showing correlation between methylation measurements for replicate samples (correlation coefficients R^2 and p-value are shown in insets).

Overall, these experiments demonstrated high correlation for methylation values for technical replicates and robust reproducibility of methylation microarray analysis.

Analytical sensitivity: lower limit of detection. The manufacturer recommended DNA amount for methylation assays is 250 ng of input for starting bisulfite conversion. Hybridization of the varying amount of DNA sample to Infinium beadchip arrays is the most straightforward method for assessing analytes sensitivity. We performed testing with increasing amount of input DNA to assess the performance of the assay. The 50, 75, 100, 150, 200, 250, 350, 500 ng of previously characterized tumor DNA was tested following full protocol starting with bisulfite conversion and finishing with hybridization to beadchips and followed by analysis in bioinformatics pipeline.

The input of 50, 75, and 100 ng tumor DNA did not produce reliable number of CpG detection, did not the pass QC test and resulted in “no match” in tumor profiling (Table 3). The 200 ng and higher amount of tumor DNA resulted in reliable number of CpG detection, acceptable QC metrics, and proper classification of the sample as high-grade glioma specimen. Interestingly, while 150 ng of input DNA produced low level of CpG detection but passed QC test and was correctly classified this specimen as high-grade astrocytoma yet with lower calibrated score of 0.4. Based on these findings, we established that required minimum amount of DNA for methylation testing is 200 ng.

Table 3. Limit of detection assessment.

Input DNA (ng)	QC2	Methylation Profiling1		
		Classifier3	Score4	MGMT promoter5
50	Fail	No match	<0.3	n/a
75	Fail	No match	<0.3	n/a
100	Fail	No match	<0.3	n/a
150	Pass	IDH glioma	0.4	Meth
200	Pass	IDH glioma, subclass high grade astrocytoma	0.99	Meth
250	Pass	IDH glioma, subclass high grade astrocytoma	0.99	Meth
350	Pass	IDH glioma, subclass high grade astrocytoma	0.99	Meth

¹Methylation Analysis of T067 with recommended CpG detection level $p=0.05$ (cut-off 96%).

²QC: pass, fail.

³Classifier score: 0-1.0

⁴MGMT promoter status: Meth – methylated, UM – unmethylated, n/a – not available.

Accuracy. The assessment of the accuracy of the methylation assay for tumor classification included testing analytical specificity and sensitivity of the assay. We used combination of the DNA isolated from normal and tumor specimen for these tests.

Analytical sensitivity: tumor content. To determine the effect of tumor percentage on the analytical sensitivity of the assay we performed experiment with various dilutions of tumor DNA in normal DNA. We selected DNA specimen which was previously characterized as glioblastoma and for which MGMT promoter methylation was determined as methylated by pyrosequencing and mixed with normal DNA at different ratios to mimic tumor content that might be observed in histological samples. Although it would be ideal to dilute tumor cells

with normal cells to determine the effect that tumor percentage has on analytical sensitivity, it is difficult to design such experiment for FFPE tissue. As a result, DNA dilutions were used as a surrogate for assessing the effect that tumor percentage has on analytical sensitivity. The data obtained from these experiments were processed in bioinformatic pipeline for QC test, tumor classification.

Sample	Normal / Tumor DNA ratio (ng)	% tumor	QC1	Methylation Profiling ²	
				MGMT promoter ³	Classifier ⁴
1	250 / 0	0	Pass	UM	NM
2	200 / 50	20%	Pass	UM	NM
3	150 / 100	40%	Pass	M	NM
4	125 / 125	50%	Pass	M	NM
5	100 / 150	60%	Pass	M	GBM (0.50)
6	50 / 200	80%	Pass	M	GBM (0.99)
7	0 / 250	100%	Pass	M	GBM (0.99)

¹QC: quality control pass or fail.

²Methylation analysis of T067 tumor DNA mixed with normal DNA at different proportion as indicated at Norm/Tumor DNA ratio column.

³MGMT promoter status: n/a – not available, M – methylated, UM – unmethylated.

⁴Tumor classification and calibrated score, range 0 - 1.0

Analytical specificity. To assess analytical specificity of the method, we performed a direct comparison of the Infinium methylation data for MGMT promoter status with pyrosequencing for the same region. We used 40 DNA samples for comparison which included diverse histological subtypes with expected different methylation status of the MGMT promoter region. All 40 samples were informative by microarray evaluation and by pyrosequencing for assessment of MGMT status, no samples failed QC. 23 out of 40 samples were negative for MGMT methylation by pyrosequencing and the same number, 23, samples were negative for methylation by microarray. We found 17 samples positive for MGMT methylation by pyrosequencing and 17 positives for methylation by microarray. However, there were two cases for which MGMT methylation status by pyrosequencing differ from microarray results. Sample T306 was negative by pyrosequencing and positive by microarray, P409 was positive by pyrosequencing and negative by microarray. These differences are due to fact that methylation status from pyrosequencing and Infinium arrays derive from different types of raw data. In pyrosequencing the data comes from single nucleotide sequencing reads while in methylation microarrays from continuous intensity values. Pyrosequencing allows detection of methylation status for 12 CpG nucleotides located in promoter region of MGMT. The results of methylation for all 12 CpG nucleotides are averaged and promoter called positive for methylation if the average is above 10% threshold. While in microarray technology the intensity values for red and green channels from methylation arrays converted into methylation signal and the promoter called methylated if the intensity is higher than the cut-off 0.3582 [20, 21]. The unbiased diagnostic accuracy of this method was estimated to 91.2% and some level of discordances in DNA methylation discovered with different analytical approaches had been described in the literature previously [22, 23]. Thus, methylation status for MGMT promoter predicted by microarray should be confirmed by another method such as pyrosequencing. Overall, we found a high correlation for MGMT promoter status evaluated

with these two independent platforms (Table 5). This is especially notable as the pyrosequencing and Infinium array DNA methylation values are derived from different types of raw data.

Table 5. Analytical specificity: MGMT promoter methylation.

	Pyrosequencing	Methylation Array
Total samples tested	40	40
Informative cases	40	36
Uninformative cases	0	0
MGMT promoter unmethylated*	23 (1)	23 (1)
MGMT promoter methylated*	17 (1)	17 (1)
Discordant cases	2	2
Analytical specificity		95%

*Numbers in parenthesis denote number of discordant cases for the class

Next, we performed direct comparison of the status for 1p and 19q chromosomal bands copy number tested by Fluorescence In Situ Hybridization (FISH) with the same regions status reported by tumor Classifier. Complete deletion of both the short arm of chromosome 1 (1p) and long arm of chromosome 19 (19q) (1p/19q co-deletion) is the molecular genetic signature of the oligodendrogliomas, a subtype of primary brain tumors accounting to approximately 10-15% of all diffuse gliomas in adults [24-26]. This molecular alteration is the result of the unbalanced whole-arm translocation between chromosome 1 and 19 with the loss of the derivative t(1p:19q), which occurs early in the pathogenesis of oligodendrogliomas. 1p/19q co-deletion is valuable diagnostic, prognostic and predictive biomarker for the management of oligodendroglial tumors [24]. We evaluated 29 specimen by methylation microarrays followed by tumor Classifier and same specimen were analyses by FISH. There were 17 cases which were negative for 1p/19q deletion by FISH and the same results were obtained by methylation Classifier. Total of 9 cases had co-deletion of 1p and 19q by FISH and these results correlated by methylation Classifier. Interestingly these 9 cases represented oligodendroglial tumors. Loss of 1p were detected by FISH in 2 cases, similar results were obtained by methylation analysis. One cases showed deletion of 19q arm by FISH and same result was confirmed by methylation analysis. One case (P738) which was positive for 19q by FISH but was negative by Classifier. Additional review revealed that 1p/19q FISH and methylation analysis was performed on a different specimen blocks. We repeated FISH on the same specimen used for methylation assay and confirmed that this specimen is negative 19q deletion. Overall, we found 100% correlation between 1p/19q status between two different platforms used for the analysis (Table 6). This is especially valuable as methylation array can provide comprehensive CNV information on whole genome level and potentially substitute FISH analysis.

Table 6. Analytical specificity: 1p/19q deletion.

	1p19q FISH	Methylation Array
Total samples tested	29	29
1p/19q intact*	17	17
1p/19q co-deleted	9	9
1p deleted*	2	2

	<table><tr><td>19q deleted*</td><td>1</td><td>1</td></tr><tr><td>Discordant cases</td><td>0</td><td>0</td></tr><tr><td>Analytical specificity</td><td>100%</td><td>100%</td></tr></table> *Numbers in parenthesis denote number of discordant cases for the class Pilot Study Summary. Our Pilot study demonstrated that the methylation data generated from the Methylation Profiling assay are extremely reproducible and reliable, and, importantly, is amenable for integration with the Classifier. The methylation data and copy number variations determined from the methylation array experiments are in concordance with the data obtained by orthogonal methods such as pyrosequencing and FISH. The calculated analytical parameters for the methylation profiling assay demonstrate 100% reproducibility and sensitivity and 98% based on the comparison of MGMT methylation status and 1p/19d data (Table 7). <table><tr><th colspan="2">Table 7. Analytical characteristics of methylation assay.</th></tr><tr><td>Parameter</td><td>Value</td></tr><tr><td>Assay reproducibility</td><td>100%</td></tr><tr><td>Analytical sensitivity</td><td>100%</td></tr><tr><td>Analytical specificity</td><td>98%</td></tr></table>	19q deleted*	1	1	Discordant cases	0	0	Analytical specificity	100%	100%	Table 7. Analytical characteristics of methylation assay.		Parameter	Value	Assay reproducibility	100%	Analytical sensitivity	100%	Analytical specificity	98%
19q deleted*	1	1																		
Discordant cases	0	0																		
Analytical specificity	100%	100%																		
Table 7. Analytical characteristics of methylation assay.																				
Parameter	Value																			
Assay reproducibility	100%																			
Analytical sensitivity	100%																			
Analytical specificity	98%																			
Diagnostic Study	Study design. The diagnostic evaluation study attempts to analyze all the parameters of the new assay that might be anticipated in clinical practice and to establish the normal and abnormal reference ranges. The experimental diagnostic sensitivity and specificity are estimates of the clinical accuracy of the test. The study comprised of 40 clinical samples including specimen from 38 retrospective diagnostic CNS tumors of different histological subtypes and control DNA. Clinical diagnoses for investigated cases were established previously and comprised of 11 different histopathologic entities from both adult and pediatric patients. The spectrum of entities was enriched for rare and difficult to diagnose cases received for referral. Histopathological evaluation was performed at the laboratory neuro-conference and included the standard molecular profiling. <i>Experimental results.</i> Methylation profiling was performed, and each case was assigned as either ‘matching to a defined DNA methylation class’ or ‘no match’ (for complete list of cases and scores see Table 9). Overall 95% of profiled samples (n=38 out of 40) matched to an established DNA methylation class with a calibrated score ≥0.3. For 36 of these (36 out of 40) results obtained by pathology and DNA methylation profiling were fully concordant. There were three cases, for which histological diagnosis were different from classification predicted based on methylation. We reviewed these cases and performed additional IHC staining to verify predicted classification. Specimen M533 was originally diagnosed in 2012 as oligodendroglioma grade II lesion but classified as astrocytoma by methylation profiling. According new 2016 WHO classification of CNS tumors the oligodendroglioma diagnosis required to demonstrate whole arm loss of 1p and 19q (1p/19q codeletion) [24]. This specimen is negative for 1p/19q deletion and should be reclassified as astrocytoma, which is in concordance with predicted classification based on methylation test. The histopathology diagnosis for specimen Q856 was anaplastic ependymoma grade III. Methylation profiling of this specimen classified it as IDH mutant glioblastoma. IHC stains for this and molecular finding of TERT, TP53 and RB1 mutations for this case were reviewed at neuropathology																			

conference and the consensus was reached that diagnosis should be revised in favor of glioblastoma. The specimen S462 diagnosed as glioblastoma were classified as “control tissue, white matter” by methylation profiling. The review of H&E slides revealed that the incorrect area which does not represent tumor was selected for DNA isolation. We repeated testing using DNA from correct area enriched with tumor cells, run methylation classifier. The new specimen was classified as glioblastoma which is in concordance with the original pathological diagnosis. Lastly, after additional review of methylation profiling results and IHC the high grade glial lesion diagnosis for specimen T302 was refined to pleomorphic xanthoastrocytoma (PXA), the diagnosis predicted by methylation classifier.

We also included in this study a DNA sample for the U87MG a widely used glioma cell line originated from glioblastoma to evaluate possible use of this specimen as a control. Methylation profiling for this sample predicted that it is a Plexus tumor. This prediction does not correlate with original source of U87 cell line. Our results show that our U87 cell line and glioblastoma samples we tested have different DNA methylation profiles. Interestingly similar conclusion that U87 cell line distributed by ATCC is not identical with original cell line established in Uppsala university using DNA fingerprinting assay was made by Allen et.al. [27]. Thus, U87 cell line is not suitable to use as a control for methylation profiling test. We plan to use previously characterized samples as controls for methylation testing while we will be searching for a proper control samples.

Overall, we found high correlation between histopathological diagnosis and classification based on methylation profiling. Interestingly, prediction made methylation classifier results in the change of the diagnoses for two specimen in favor of the diagnoses predicted by methylation classifier. Interestingly, methylation profiling test can detect errors when wrong specimen was taken for testing and should aid in reduction of such human errors.

Table 8. Summary of the diagnostic study

Specimen	Clinical History	Classifier	Calibrated Score	Consensus
S728	Meningioma	Meningioma	0.99	Yes
S852	Meningioma	Meningioma	0.99	Yes
S918	Anaplastic oligodendroglioma	IDH glioma, subclass 1p/19q co-deleted oligodendroglioma	0.99	Yes
S959	Meningioma	Meningioma	0.99	Yes
T043	Ependymoma, spinal	Ependymoma, spinal	0.99	Yes
T067	Glioblastoma	IDH glioma, subclass high grade astrocytoma	0.99	Yes
T210	Meningioma	Meningioma	0.99	Yes
T306	High grade astrocytic lesion	Glioblastoma, IDH wildtype, subclass mesenchymal	0.85	Yes
M533	Oligodendroglioma	IDH glioma, subclass astrocytoma	0.78	Yes ¹

	P409	Anaplastic astrocytoma	IDH glioma, subclass high grade astrocytoma	0.68	Yes
	P589	Oligodendroglioma	IDH glioma, subclass 1p/19q co-deleted oligodendroglioma	0.99	Yes
	P738	Glioblastoma	IDH glioma, subclass high grade astrocytoma	0.98	Yes
	P850	Oligodendroglioma	IDH glioma, subclass 1p/19q co-deleted oligodendroglioma	0.99	Yes
	P879	Ependymoma	Ependymoma, spinal	0.99	Yes
	Q060	Chordoma	Chordoma	1.00	Yes
	Q103	Medulloblastoma	Medulloblastoma, subclass group IV	0.99	Yes
	Q312	Glioblastoma	Glioblastoma, IDH wildtype, IDH wildtype, subclass mesenchymal	0.76	Yes
	Q853	Oligodendroglioma	IDH glioma, subclass 1p/19q co-deleted oligodendroglioma	0.99	Yes
	Q856	Ependymoma, anaplastic	Glioblastoma, IDH wildtype, subclass mesenchymal	0.40	Yes ¹
	Q859	Oligodendroglioma	IDH glioma, subclass 1p/19q co-deleted oligodendroglioma	0.99	Yes
	Q992	Glioblastoma	Glioblastoma	0.46	Yes
	R084	Astrocytoma	IDH glioma, subclass astrocytoma	0.96	Yes
	U87	GBM cell line	Plexus Tumor	0.41	Yes
	S274	Glioblastoma	Glioblastoma, IDH wildtype	0.99	Yes
	R186	Hemangiopericytoma	Solitary fibrous tumor / hemangiopericytoma	0.99	Yes
	T211	Glioblastoma	Glioblastoma, IDH wildtype	0.99	Yes
	R085	Hemangiopericytoma	Solitary fibrous tumor / hemangiopericytoma	0.99	Yes

	R418	Anaplastic oligodendroglioma	Glioma, IDH mutant, subclass 1p/19q co-deleted oligodendroglioma	0.99	Yes
	S457	Glioblastoma	Glioblastoma, IDH wildtype, subclass mesenchymal	0.57	Yes
	R314	Glioblastoma	Glioblastoma, IDH wildtype	0.99	Yes
	T215	Glioblastoma	Glioblastoma, IDH wildtype	0.38	Yes
	T213	Ependymoma	Paraganglioma, spinal non-CIMP	1.00	Yes
	S462	Glioblastoma	Control tissue, white matter	0.77	Yes ³
	T212	Glioblastoma	Glioblastoma, IDH wildtype	0.99	Yes
	S353	Malignant sarcomatoid neoplasm, History of Glioblastoma	Glioblastoma, IDH wildtype	0.38	Yes
	R954	Glioblastoma	Glioblastoma, IDH wildtype, subclass mesenchymal	0.96	Yes
	T302	HG Glial lesion	Pleomorphic Xanthoastrocytoma (PXA)	0.31	Yes ⁴
	R267	Pineoblastoma	Pineal parenchymal tumor	0.98	Yes
	¹M533 - methylation classification was confirmed after IHC review, diagnosis is revised in favor of oligodendroglioma according new WHO ²Q956 – methylation classification was confirmed after IHC review, diagnosis is revised in favor of glioblastoma ³S462 – methylation classification confirmed after analysis of DNA from correct tumor area ⁴T302 – methylation classification helped to refine the diagnosis for this specimen, the Dx was revised after IHC review. Alternative proficiency assessment by exchanging samples with another laboratory is an efficient method to verify testing in clinical laboratories. The 40 samples of DNA we sent to NYU Langone Health center for methylation testing and classification (see Table 9 for specimen histological subtypes). This laboratory is CLIA certified and CAP accredited and recently validated methylation testing. NYU laboratory performed complete methylation workflow and bioinformatic analysis using Classifier at their center. The results of the testing and tumor classification are presented in Table 10.				

Table 9. Histological subtypes of tissue samples used for sample exchange study.

Clinical Diagnosis	Number of cases
Astrocytoma	3
Chordoma	1

Ependymoma	4
Glioblastoma	13
Gliosarcoma	1
Hemangiopericytoma	2
Medulloblastoma	1
Meningioma	4
Oligodendroglioma	7
Pineoblastoma	1
Pleomorphic xanthoastrocytoma (PXA)	1
Control tissue	2
TOTAL	40

Overall, the results obtained by NYU laboratory were in ... concordance with the results obtained in our laboratory for tumor Classification, CNV and MGMT promoter assessment. Thus, our evaluation of methylation testing and classification with CNS classifier demonstrated correlation with histopathological diagnosis and results obtained by another laboratory performing similar testing.

Table 10. Methylation classification results for sample exchange study.

Specimen ID	Histological Dx	NCI Classifier result	NCI , C-score*	NY U Classifier result	NY U, C-score*	Consensus
T306	Astrocytic lesion, high grade	Glioblastoma, IDH wildtype, subclass mesenchymal	0.85			
R084	Astrocytoma	IDH glioma, subclass astrocytoma	0.96			
P409	Astrocytoma, anaplastic	IDH glioma, subclass high grade astrocytoma	0.68			
Q060	Chordoma	plexus tumor, subclass pediatric B	0.59			
P879	Ependymoma	Ependymoma, spinal	0.99			
Q856	Ependymoma	Glioblastoma, IDH wildtype, subclass mesenchymal	0.4			
T213	Ependymoma	Paranglioma, spinal non-CIMP	0.999			
T043	Ependy-moma, spinal	Ependymoma, spinal	0.99			
P738	Glioblastoma	IDH glioma, subclass high grade astrocytoma	0.98			
Q312	Glioblastoma	Glioblastoma, IDH wildtype, IDH wildtype, subclass mesenchymal	0.76			
Q992	Glioblastoma	No matching methylation classes	0			

R314	Glioblastoma	Glioblastoma, IDH wildtype	0.99
R954	Glioblastoma	Glioblastoma, IDH wildtype, subclass mesenchymal	0.96
S274	Glioblastoma	Glioblastoma, IDH wildtype	0.99
S457	Glioblastoma	Glioblastoma, IDH wildtype, subclass mesenchymal	0.57
S462	Glioblastoma	Control tissue, white matter	0.77
T067	Glioblastoma	IDH glioma, subclass high grade astrocytoma	0.99
T211	Glioblastoma	Glioblastoma, IDH wildtype	0.99
T212	Glioblastoma	Glioblastoma, IDH wildtype	0.99
T215	Glioblastoma	No matching methylation classes	<0.3
S353	Glioblastoma	Glioblastoma, IDH wildtype	0.38
T599	Gliosarcoma, G34R-mutant	Glioblastoma, IDH wildtype, H3.3 G34 mutant	0.77
R085	Hemangiopericytoma	Solitary fibrous tumor / hemangiopericytoma	0.99
R186	Hemangiopericytoma	Solitary fibrous tumor / hemangiopericytoma	0.99
Q103	Medullo-blastoma	Medulloblastoma, subclass group 4	0.99
S728	Meningioma	Meningioma	0.99
S852	Meningioma	Meningioma	0.99
S959	Meningioma	Meningioma	0.99
T210	Meningioma	Meningioma	0.99
M533	Oligodendro-glioma	IDH glioma, subclass astrocytoma	0.78
P589	Oligodendro-glioma	IDH glioma, subclass 1p/19q codeleted oligodendroglioma	0.99
P850	Oligodendro-glioma	IDH glioma, subclass 1p/19q codeleted oligodendroglioma	0.99
Q853	Oligodendro-glioma	IDH glioma, subclass 1p/19q codeleted oligodendroglioma	0.99
Q859	Oligodendro-glioma	IDH glioma, subclass 1p/19q codeleted oligodendroglioma	0.99
R418	Anaplastic oligodendro-glioma	Glioma, IDH mutant, subclass 1p/19q codeleted oligodendroglioma	0.99
S918	Anaplastic oligodendro-glioma	IDH glioma, subclass 1p/19q codeleted oligodendroglioma	0.99
R267	Pineoblastoma	Pineal parenchymal tumor	0.98
T302	PXA, pleomorphic xantho-astrocytoma	PXA, pleomorphic xanthoastrocytoma	<0.3
NA19741	Control, normal DNA	Plexus Tumor	0.36
NA20510	Control, normal DNA	Plasmacytoma	0.82

	*C-score: Calibrated score <i>Final evaluation.</i> We have performed comprehensive validation of the methylation profiling and CNS Classification algorithm. Results of testing were confirmed by pathological diagnosis and testing performed by independent laboratory for Methylation Classifier in whole and independent such as FISH for 1p/19q deletion and pyrosequencing for MGMT promoter status. There were no false positive cases. This result allows the <i>diagnostic specificity</i>, which accounts for the percentage of false positive cases identified in assay, i.e. assesses probability that the test is negative if patient does not have disease; $P[T^- D^-]$. The calculated diagnostic specificity is equal to 100%. All positive cases diagnosed by histopathological studies were identified and classified our study. This result allows to assess the <i>diagnostic sensitivity</i>, which is a percentage of true positive cases, identified by the assay as positive, i.e. probability that the test is positive if the patient has disease; $P[T^+ D^+]$. The calculated diagnostic sensitivity is equal to 100%.
Summary of Methylation Classifier Validation	The methylation classifier test is designed to classify tumors based on DNA methylation profiling using machine-learned algorithm. This test is useful for the classification of CNS tumors as it allows high level of standardization, which has a great promise to reduce substantial inter-observer variability perceived in current CNS tumor diagnostics. The validation study allowed us to obtain the analytic and diagnostic characteristics of methylation classifier test. This test permits robust detection of whole genome methylation profile, copy number profile, and MGMT promoter methylation status based on Infinium MethylationEPIC assay combined with CNS Classifier. During this study we observed 40 cases for which pathology and methylation profiling were concordant. For two cases the methylation class was not in accordance with pathological diagnosis. However, a subsequent pathology review of additional IHC and molecular testing resulted in revision of diagnosis in favor of predicted methylation class. Based on testing of 40 specimen set we accepted the commonly used cut-off point ≥ 0.5 for calibrated score threshold [28]. This diagnostic study demonstrated 100% concordance of the methylation profiling results with histopathological diagnosis as well as ... correlation of our laboratory results with results obtained at another reference laboratory. We also demonstrated 100% correlation for the 1p/19q and MGMT promoter status predicted by methylation Classifier with the results obtained using orthogonal methods such as FISH for 1p/19q and pyrosequencing for MGMT promoter status. Based on these findings, the methylation-based classification test demonstrates acceptable performance and is approved for implementation in clinical practice for patient testing. SHH Subtyping Validation Methylation profiling is considered the definitive method for subtyping of medulloblastoma in the current WHO ‘Blue Book’ for the classification of CNS neoplasms. To validate its use at the Laboratory of Pathology at the NCI, we profiled by methylation a set of 95 medulloblastomas that had been previously subtyped using orthogonal methods (e.g. immunohistochemistry and/or DNA sequencing). These 85 cases were sent to the NCO for consultation/methylation profiling from outside institutions and had been subtyped into one of 3 major classes (SHH, WNT, non-SHH, non-WNT) at the outside institution using either immunohistochemistry or sequencing. In this cohort of 95 cases the subtype determination before methylation

	profiling was used as the gold standard with which methylation results were compared. Of the 95 cases, the outside subtype determination included SHH (43 cases); WNT (8 cases); non-SHH, non-WNT (44 cases). Methylation profiling was performed and the DKFZ version 11 classifier results, compared to the gold standard, results of the 3 X3 table are below: <table border="1"> <tr> <th colspan="2"></th><th colspan="3">Methylation-based classification (NCI)</th></tr> <tr> <th colspan="2"></th><th>SHH</th><th>WNT</th><th>non-SHH, non-WNT</th></tr> <tr> <th rowspan="3">Outside (pre-methylation) subtype determination</th><th>SHH</th><td>43</td><td>0</td><td>0</td></tr> <tr> <th>WNT</th><td>0</td><td>8</td><td>0</td></tr> <tr> <th>non-SHH, non-WNT</th><td>0</td><td>1</td><td>43</td></tr> </table> Results show a highly significant (Fisher's exact test $p < 0.01$) relationship of methylation-based classification with the gold standard, including all 43 cases of SHH medulloblastoma being concordantly classified as such by 2 methods. Findings indicate that medulloblastoma subtyping can be accurately performed using methylation profiling in the CLIA facility in the Laboratory of Pathology at the NCI.						Methylation-based classification (NCI)					SHH	WNT	non-SHH, non-WNT	Outside (pre-methylation) subtype determination	SHH	43	0	0	WNT	0	8	0	non-SHH, non-WNT	0	1	43
		Methylation-based classification (NCI)																									
		SHH	WNT	non-SHH, non-WNT																							
Outside (pre-methylation) subtype determination	SHH	43	0	0																							
	WNT	0	8	0																							
	non-SHH, non-WNT	0	1	43																							
Pathology Report Disclaimer	The following disclaimer will be follow each pathology report when the methylation-based classification results will be reported: "Analyte specific reagents (ASR) are used in many laboratory tests necessary for standard medical care and generally do not require U.S. Food and Drug Administration (FDA) approval or clearance. This analysis is a supplement to the diagnosis of the requesting pathologist and does not represent final diagnosis. This test was developed, and its performance characteristics were determined by the Chromosome Pathology Section, Laboratory of pathology, CCR NCI. It has not been cleared or approved by the U.S. Food and Drug Administration. The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigational or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA-88) as qualified to perform high complexity clinical laboratory testing".																										
References	Uncategorized References Baylin SB, Jones PA: Epigenetic Determinants of Cancer. <i>Cold Spring Harb Perspect Biol</i> 2016, 8(9).																										

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Table and Figure legends	Table 1. Infinium methylation controls.

	Table 2. Reproducibility analysis using replicate samples*. Table 3. Limit of detection assessment. Table 4. Analytical sensitivity – tumor content. Table 5. Analytical specificity: MGMT promoter methylation. Table 6. Analytical specificity: 1p/19q deletion. Table 7. Analytical characteristics of methylation assay. Table 8. Histological subtypes of tissue samples used for diagnostic study. Table 9. Summary of the diagnostic study Table 10. Sample exchange experimental results Figure 1. The Infinium MethylationEPIC beadchip coverage throughout gene regions. Figure 2. Reproducibility of methylation assay. Scatter plots showing correlation between methylation measurements for replicate samples (correlation coefficients R² and p-value are shown in insets).
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APPENDIX D3 Medulloblastoma (MB) Subgrouping Validation – NCH

Background and Clinical Utility	Clinical Utility Histopathological features have been traditionally used to classify tumors using World Health Organization (WHO) criteria. Tumor grading serves as a means to characterize cellularity, mitotic activity, pleomorphism, necrosis, and endothelial proliferation, predicting the biological behavior of a neoplasm, as well as influencing the choice of medical treatments (Louis et al. 2016. WHO). However, the assessment of tumors with atypical/nonclassical morphologies can often be challenging and subjective. Inter-observer variability in the diagnosis of brain tumors has been documented in several studies (Gupta <i>et al.</i> 2005, <i>Am J Clin Pathol</i>; Gilles <i>et al.</i> 2008, <i>Pediatr Dev Pathol</i>; van den Bent, 2010 <i>Acta Neuropathol</i>). Central nervous system (CNS) tumors are a type of neoplasm arising from tissues of the brain and/or spinal cord, and are comprised of various entities with specific morphologies. The 2016 edition of the WHO classification of CNS tumors updated the classification by incorporating molecular and cytogenetic abnormalities in several CNS tumor entities (Louis et al. WHO. 2016). Recently, several groups have demonstrated the potential diagnostic and prognostic value in DNA-methylation-based classification for patients diagnosed with CNS tumors (Nassiri et al. 2019. <i>Neuro Oncol</i>; Karimi <i>et al.</i> 2019. <i>Clin Epigenetics</i>; Capper <i>et al.</i> 2018, <i>Nature</i>). Key to DNA-based methylation array profiling is use of a comprehensive machine-learning approach applying a random forest classifier to calculate probabilities of the family and class of the tumor. This classifier has been developed by the German Cancer Research Center (DKFZ), German Consortium for Translational Cancer Research (DKTK), and University Hospital Heidelberg (https://www.molecularneuropathology.org/mnp). Specifically, the Capper <i>et al.</i> study showed that data from a DNA methylation-based array, used in concert with a methylation profiling classifier to predict disease-entity, led to a change of diagnosis for approximately 12% of CNS tumor cases (Capper <i>et al.</i> 2018, <i>Nature</i>). Thus, through prior study, it has become evident that the combination of histology, genetics, and DNA methylation can increase the sensitivity of CNS tumor diagnostics. Additionally, <i>MGMT</i> methylation status can also be evaluated using the classifier. <i>MGMT</i> is a DNA repair enzyme, which has the capability to rescue tumor cells from agent-induced damage, leading to resistance to chemotherapy with alkylating agents. Methylation of the <i>MGMT</i> promoter region results in decreased <i>MGMT</i> protein expression, reduced DNA repair activity, and potential increased sensitivity to therapy. For patients with glioblastomas, the presence of <i>MGMT</i> promoter methylation has been shown to be an independent favorable prognostic factor and a strong predictor of responsiveness to chemotherapy. The goal of this validation study is to establish assay and diagnostic performance of the Illumina MethylationEPIC 850k array for patients with CNS tumors. Additional information about the DKFZ CNS methylation profiling classifier can be found here: https://www.molecularneuropathology.org/mnp Current State of Test Options As mentioned above, CNS tumor subtyping has been historically evaluated by histology, including immunohistochemical studies (Louis et al. 2016. WHO). Within the last 10 years, molecular features have been incorporated to help refine the molecular subtyping of CNS
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tumors such as: *IDH*-wildtype glioblastoma, *IDH*-mutant glioblastoma, H3 K27M-mutant diffuse midline glioma, *WNT*-activated medulloblastoma, and others. These molecular features are clinically significant because they can help to refine the prognosis or treatment options for patients.

DNA-based methylation profiling of CNS tumors could further refine the histologically challenging subtypes (Nassiri et al. 2019. *Neuro Oncol*; Karimi *et al.* 2019. *Clin Epigenetics*; Capper *et al.* 2018, *Nature*). Notably, it was demonstrated that DNA-based methylation profiling would produce reliable results even from small samples and poor-quality material (Hovestadt, et al. *Acta Neuropathol* 2013). Few laboratories have started to implement this technology in their institutions to assist in CNS tumor subtyping.

Moreover, for the MGMT methylation assay, methylation status has been historically determined by methylation-sensitive primers and PCR and performed as a stand-alone assay.

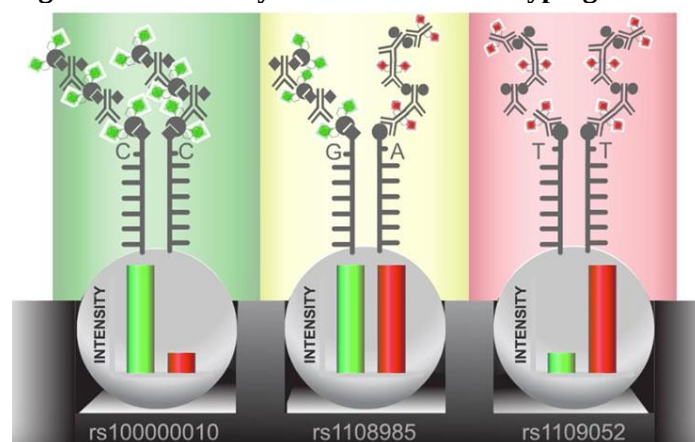
Given the past studies on the clinical utility of these assays, it is evident that the implementation of DNA-based methylation profiling, along with the MGMT methylation status, is clinically beneficial for patients with CNS tumors.

Test Method

Experimental Approach

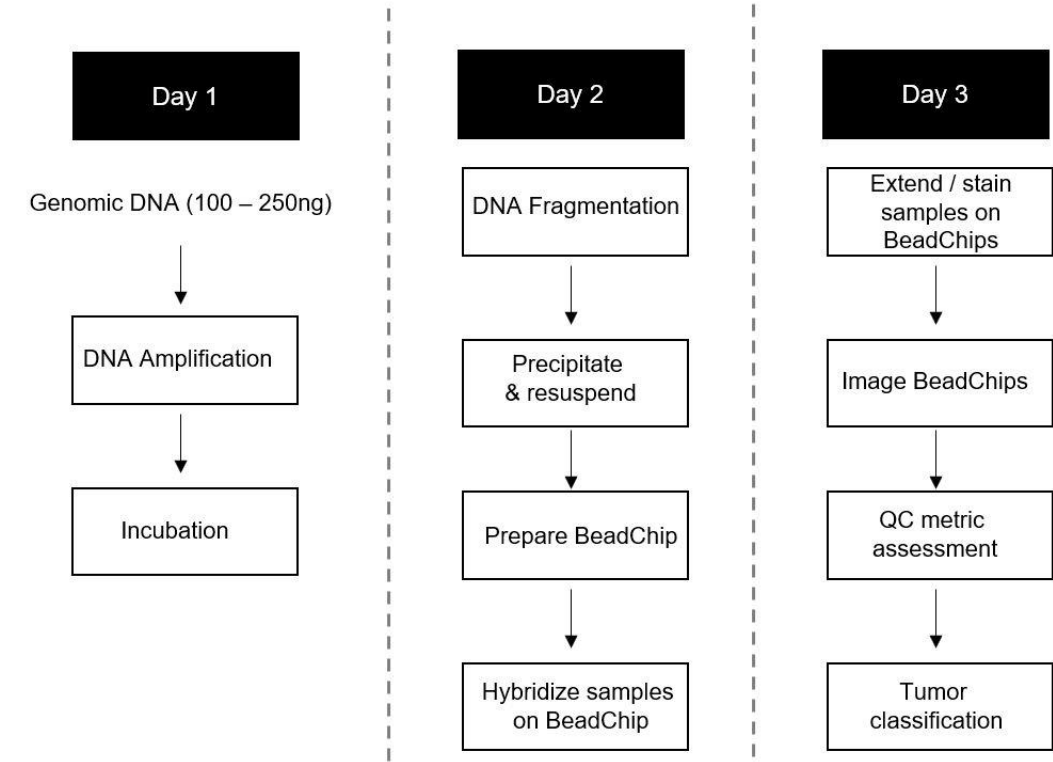
This test will be carried out using the Illumina Infinium MethylationEPIC 850K Microarray kit, and the methylation profiling classifier developed by the [German Cancer Research Center \(DKFZ\)](#), [German Consortium for Translational Cancer Research \(DKTK\)](#), and [University Hospital Heidelberg](#) (<https://www.molecularneuropathology.org/mnp>). The Illumina methodology uses BeadArray technology to detect hundreds of thousands of genotypes for a single sample at once. As the DNA fragments (extracted from CNS tumor specimens) pass over the BeadChip, each probe binds to a complementary sequence in the sample DNA. Allele specificity is conferred by a single base extension that incorporates one of four labeled nucleotides. When excited by a laser, the nucleotide label emits a signal. The intensity of that signal conveys information about the allelic ratio at that locus (**Figure 1**).

Figure 1. BeadArray Detection for Genotyping



The Infinium MethylationEPIC microarray chip interrogates over 850,000 methylation sites quantitatively across the genome at single-nucleotide resolution. The coverage of this platform includes CpG islands, non-CpG methylated sites identified in human stem cells, differentially methylated sites identified in tumor versus normal, FANTOM5 enhancers, ENCODE open chromatin and enhancers, miRNA promoter regions, etc.

Figure 2. Overview of Illumina Infinium EPIC protocol



The protocol is summarized in the following steps as demonstrated in **Figure 2**.

Day 1

1. Sample DNA undergoes bisulfite conversion using the EZ DNA Methylation Gold Kit. The treatment of DNA with bisulfite will convert unmethylated cytosine residues to uracil but leaves the methylated cytosine unaffected.
2. DNA is then amplified by PCR and incubated overnight.

Day 2

1. DNA is enzymatically fragmented.
2. DNA is then alcohol precipitated and resuspended.
3. Sample DNA is hybridized to the microarray BeadChips.

Day 3

1. Samples undergo enzymatic base extension and fluorescent staining
2. BeadChips are imaged by iScan System

13.4.1 **Figure 3: Bioinformatic Analysis Approach**

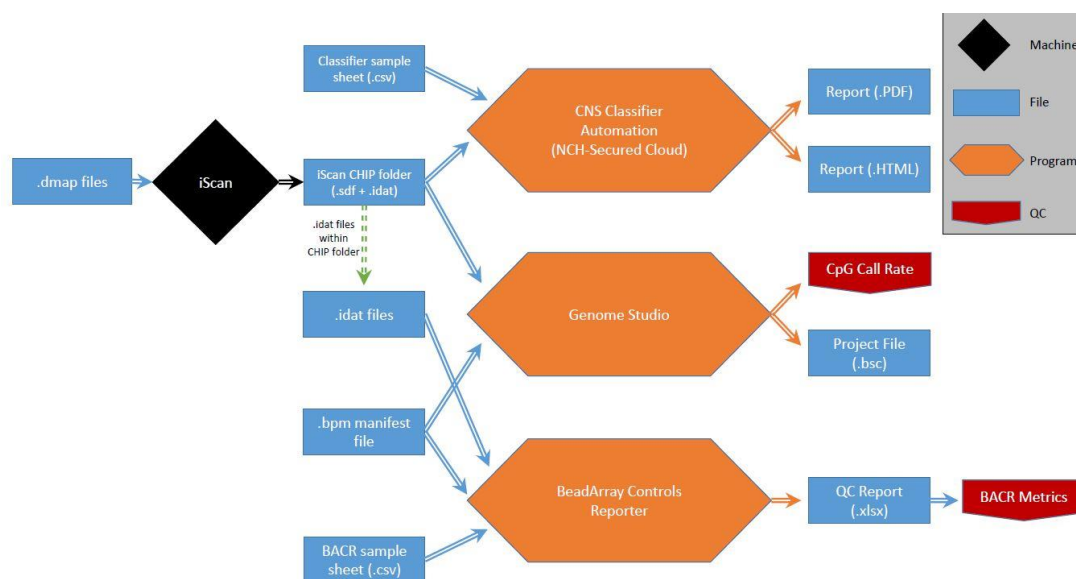


Figure 3 summarizes the bioinformatic workflow for this DNA-based methylation array assay. All files are retained for a minimum of 2 years, per CAP guidelines.

In summary, CNS tumor classification is performed by processing raw IDAT files through the CNS Classifier algorithm (version 11b6), which was developed by the German Cancer Research Center (DKFZ), German Consortium for Translational Cancer Research (DKTK), and University Hospital Heidelberg (<https://www.molecularneuropathology.org/mnp>). A local version of the CNS Classifier R package is utilized, and it is invoked when a sample sheet is added by the technologist that matches the unique IDAT barcode, which is output by the iScan. This process allows for automated invocation of a docker container that runs the CNS Classifier pipeline in the NCH-secured cloud. PDF and html reports are generated for each sample, which include classification, score, clustering by tSNE, and copy number plots, are output to a dedicated and secure S3 folder for each array that is run.

CpG call rate is used as a quality control metric for the methylation assay. This metric is a calculation of the percentage of CpG locations that were successfully detected by the assay. The Illumina GenomeStudio V2011.1 utilizes the contents of the output folder produced by the iScan, including the chip specific .sdf file and the patient specific .idat files and creates a GenomeStudio project file. The program displays the number of CpG sites detected on the array with a p-value of 0.01 and with a p-value of 0.05. The CpG call rate is calculated by dividing the number of CpG sites detected with a p-value of 0.05 by the total number of detectable CpG sites (865,918). For a passing metric, the CpG call rate must be above 96% (detected CpG with a p-value of 0.05 above 831,281) for frozen samples or above 90% (detected CpG with a p-value of 0.05 above 779,326) for FFPE samples. GenomeStudio also displays graphical outputs of QC data that can be reviewed for a more in-depth analysis if something is flagged in the BeadArray Controls reporter.

The Illumina BeadArray Controls Reporter (BACR) V1.1.0 is a program used to normalize and analyze 21 different QC metrics of the array. The BACR requires the input of the .idat files produced by the iScan, the .bpm manifest file downloaded from Illumina, and a .csv sample sheet created by the tech to outline which samples correspond to which .idat files. A .xlsx (Excel) file is output that contains a table of the samples and their normalized QC output. Any metrics that do not meet the input cut-offs are flagged for further review.

Acceptable Specimen Type

Formalin-fixed, paraffin embedded (FFPE) or frozen CNS cancer tissue with a tumor cellularity, as documented by a pathologist, of greater or equal to 60% tumor nuclei is acceptable for this assay. This assay requires an input of 100ng DNA extracted from the tumor block in the form of FFPE scrolls, scrapings, or macro-dissected tissue, as appropriate. No matched normal sample will be assayed.

Testable Regions

The classifier uses the methylation data generated from the >850,000 CpG probes from the Illumina methylation BeadChip array. The array probes cover regions throughout the genome, including CpG islands, non-CpG methylated sites identified in human stem cells, differentially methylated sites identified in tumor versus normal, FANTOM5 enhancers, ENCODE open chromatin and enhancers, and miRNA promoter regions.

Demonstration of Testing Performance

Illumina MethylationEPIC array QC

Independent from the analyses directed toward the CNS subtype classification based on methylation, we systematically evaluated the microarray performance using the Illumina BACR software, which is designed to analyze the sample controls for Illumina BeadArray products. The input for BACR are the IDAT files with the output representing an Excel file that summarizes the results for the control probes. Below are categories that are used for quality assessment. The detailed definition of each metric can be found in the Illumina BACR user guide. (Of note, C/G are in the green channel; A/T are in the red channel.)

As derived on the basis of the manufacturer's recommendation, **the clinical QC metrics established in this validation include:**

1. ≤ 1 failed metric for the Bisulfite conversion controls (shaded in gray below)
2. All nonpolymorphic controls passing (shaded in gray below)
3. A CpG call rate above 96% (detected CpG with a p-value of 0.05) for frozen samples or above 90% (detected CpG with a p-value of 0.05) for FFPE.

Below are the cutoff values for each control (default set by manufacturer)

Categories:	Restoration (only FFPE samples)	Staining Green	Staining Red	Extension Green	Extension Red	Hybridization High Medium	Hybridization Medium Low	Target Removal1	Target Removal2
Minimum Threshold:	> 0	5	5	5	5	1	1	1	1

Bisulfite Conversion1 Background Green	Bisulfite Conversion1 Background Red	Bisulfite Conversion1 Background Green	Bisulfite Conversion1 Background Red	Bisulfite Conversion2 Background	Specificity1 Green	Specificity1 Red	Specificity2	Specificity2 Background	NonPolymorphic Green	NonPolymorphic Red
1	1	1	1	1	1	1	1	1	5	5

1. The **restoration control** identifies success of the FFPE restoration chemistry. For a frozen sample, this metric would be 0.
2. The **staining controls** are used for examining the efficiency of the staining step in both the red and green channels. These controls are independent of the hybridization and extension steps.
3. The **extension controls** test the extension efficiency of A, T, C, and G nucleotides from a hairpin probe, and are therefore sample independent.
4. The **hybridization controls** test the overall performance of the Infinium Assay using synthetic targets instead of amplified DNA. The synthetic targets are present in the Hybridization buffer (RA1) at 3 levels, monitoring the response from high-concentration, medium concentration, and low concentration.
5. **Target removal controls** test the efficiency of the stripping step after the extension reactions.
6. **Bisulfite conversion 1 controls** use Infinium I probe design and allele-specific single base extension to monitor efficiency of bisulfite conversion. If the bisulfite conversion reaction was successful, the Converted probes matches the converted sequence and get extended. If the sample has unconverted DNA, the Unconverted probes get extended.
7. **Bisulfite conversion 2 controls** use Infinium II probe design. If the bisulfite conversion reaction was successful, the “A” base gets incorporated and the probe has intensity in the red channel. If the sample has unconverted DNA, the “G” base gets incorporated across the unconverted cytosine, and the probe has elevated signal in the green channel.
8. **Specificity controls** are designed to monitor potential nonspecific primer extension for Infinium I and II assay probes. Specificity controls are designed against nonpolymorphic T sites.
9. **Nonpolymorphic controls** test the overall performance of the assay from amplification to detection, by querying a particular base in a nonpolymorphic region of the genome. One nonpolymorphic control has been designed for each of the 4 nucleotides (A, T, C and G). Of note, C/G are in the green channel; A/T are in the red channel.)
 1. In the green channel, the lowest intensity of C or G is always greater than the highest intensity of A or T.
 2. In the red channel, the lowest intensity of A or T is always greater than the highest intensity of C or G.

An example of a sample that passed QC.

Categories:	Restoration	Staining Green	Staining Red	Extension Green	Extension Red	Hybridization High Medium	Hybridization Medium Low	Target Removal1	Target Removal2
A sample that passes QC	0.0	56.3	35.6	87.8	16.2	1.5	1.7	28.6	17.5

Bisulfite Conversion1 Background Green	Bisulfite Conversion1 Background Red	Bisulfite Conversion1 Background Green	Bisulfite Conversion1 Background Red	Bisulfite Conversion2 Background	Specificity1 Green	Specificity1 Red	Specificity2	Specificity2 Background	NonPolymorphic Green	NonPolymorphic Red	CpG Call Rate
3.5	11.6	3.4	8.7	2.2	9.3	4.0	42.5	10.1	14.5	18.8	0.9983

An example of a sample with staining error.

Highlighted represents failed QC (for our assay, only Bisulfite conversion probes, nonpolymorphic probes and CpG call rate are used). As demonstrated below, inadequate

staining would lead to failure in other parts of quality metrics (extension, hybridization, conversion, call rate, etc).

Categories:	Restoration	Staining Green	Staining Red	Extension Green	Extension Red	Hybridization High Medium	Hybridization Medium Low	Target Removal1	Target Removal2
A sample with insufficient staining	0.6	0.8	27.7	0.5	16.7	0.3	2.3	24.9	27.6

Bisulfite Conversion1 Background Green	Bisulfite Conversion1 Red	Bisulfite Conversion1 Background Red	Bisulfite Conversion2	Bisulfite Conversion2 Background	Specificity1 Green	Specificity1 Red	Specificity2	Specificity2 Background	NonPolymorphic Green	NonPolymorphic Red	CpG Call Rate
23.5	0.9	24.5	2.2	26.9	0.7	0.6	2.5	33.2	0.9	0.6	0.2386

Below is an example of a sample that was not undergone bisulfite conversion. Highlighted represents failed QC.

Categories:	Restoration	Staining Green	Staining Red	Extension Green	Extension Red	Hybridization High Medium	Hybridization Medium Low	Target Removal1	Target Removal2
A sample without bisulfite conversion	0.0	111.4	23.9	59.4	12.2	1.5	1.9	18.5	13.6

Bisulfite Conversion1 Background Green	Bisulfite Conversion1 Red	Bisulfite Conversion1 Background Red	Bisulfite Conversion2	Bisulfite Conversion2 Background	Specificity1 Green	Specificity1 Red	Specificity2	Specificity2 Background	NonPolymorphic Green	NonPolymorphic Red	CpG Call Rate
0.1	0.0	0.3	0.0	0.2	0.6	1.0	1.5	22.3	0.5	0.7	0.2549

Array performance of frozen tissues

To test whether our lab can process samples meeting sufficient QC metrics, we ran 27 frozen CNS samples across 10 different array chips in accordance with the manufacturer's instructions.

Required clinical QC metrics:

1. ≤ 1 failed metric for the Bisulfite conversion controls
2. All nonpolymorphic controls passing
3. A CpG call rate above 96% (detected CpG with a p-value of 0.05) for frozen samples or above 90% (detected CpG with a p-value of 0.05) for FFPE.

All the samples listed in the table below passed the required clinical QC metrics.

	Sample Name	Sentrix Barcode	Sentrix Position	Bisulfite Conversion on1 Green	Bisulfite Conversion on1 Background Green	Bisulfite Conversion on1 Red	Bisulfite Conversion on1 Background Red	Bisulfite Conversion on2	Bisulfite Conversion on2 Background	NonPoly morphic Green	NonPoly morphic Red	CpG Call Rate
1	FAM0122_S-190827-00008	203550290083	R01C01	9.7	2.6	6.1	1.5	12.1	2.1	14.4	12.5	99.95%
2	FAM0121_S-190820-00062	203550290083	R02C01	5.7	1.4	5.5	1.4	8.1	1.4	12.5	13.3	99.91%
3	FAM0116_S-190808-00043	203550290083	R03C01	8.2	2.0	5.7	1.4	9.1	1.6	12.8	14.1	99.96%
4	CH_FAM0005_M18-1884_Z0149	203550290083	R04C01	11.7	2.8	5.4	1.4	10.8	1.9	15.7	11.5	99.95%
5	JL-17-029_H6279	203550290083	R05C01	12.0	2.4	5.4	1.3	7.1	1.3	11.9	16.3	99.95%
6	JL-17-013_H3182	203550290083	R06C01	12.5	2.5	4.4	1.0	9.6	1.6	13.4	15.1	99.97%
7	JL-17-018_H4483	203808720074	R01C01	11.8	3.2	6.7	1.6	9.1	1.7	12.1	16.6	99.93%
8	FAM0124_S-190904-00019	203806760115	R01C01	10.9	2.7	7.3	1.4	10.7	1.8	17.4	15.9	99.80%
9	FAM0138_S-191014-00064	203806760142	R06C01	5.9	1.4	7.2	1.7	8.1	1.7	13.3	17.5	99.93%
10	FAM0125_S-190904-00017	203806760142	R07C01	8.9	2.1	6.4	1.5	7.0	1.6	13.6	18.0	99.97%
11	CH_FAM0064_M18-6355_Z0918	203806760103	R04C01	11.1	3.3	7.1	2.1	6.8	2.2	14.9	18.1	99.60%
12	CH_FAM0029_M18-3300_Z0355	203806760112	R03C01	13.5	3.8	7.9	2.2	8.4	2.3	14.3	17.8	99.93%
13	CH_FAM0021_M18-2562_Z0244	203806760112	R04C01	14.0	3.9	7.8	2.2	6.3	1.9	11.6	18.9	99.97%
14	FAM0126_S-191001-00076	203806760112	R05C01	12.2	3.3	7.9	2.2	6.3	2.0	15.9	17.1	99.95%
15	JL-17-004_H2218	203806760112	R06C01	13.4	3.7	7.9	2.2	6.9	2.0	15.1	22.8	99.93%
16	JL-17-006_H2315	203806760112	R07C01	14.6	4.1	11.0	3.1	6.3	2.1	17.4	17.9	99.90%
17	CH_FAM0142_S-191105-00045	203991470112	R02C01	9.6	2.5	5.1	1.4	7.9	1.7	11.5	15.8	99.93%
18	JL-17-027_H6199	203991470112	R05C01	8.8	2.3	5.0	1.4	6.7	1.5	15.9	11.2	99.87%
19	JL-18-033_H6876	203991470112	R07C01	9.2	2.3	7.0	1.9	5.4	1.6	13.2	18.6	99.92%
20	CH_FAM0147_S-191118-00007	203991470101	R05C01	7.3	1.4	3.8	1.0	9.1	1.5	14.3	14.5	99.95%
21	CH_FAM0131_S-191108-00007	203981500153	R02C01	8.2	1.8	4.4	1.1	7.2	1.4	10.6	17.9	99.92%
22	CH_FAM0155_S-191205-00059	203981500153	R05C01	6.9	1.5	4.7	1.2	8.9	1.7	11.2	16.1	99.97%
23	JL-17-032_Z1420	203981500153	R07C01	9.3	1.7	5.6	1.5	7.5	1.2	11.9	15.4	99.96%
24	JL-17-032_Z1441	203982200004	R01C01	6.9	2.2	6.2	1.9	5.7	1.5	15.8	15.3	99.95%
25	CH_FAM0139_S-191108-00003	203982200004	R04C01	4.6	1.1	4.7	1.2	7.3	1.7	12.1	19.8	99.97%
26	CH_FAM0139_S-191112-00020	203982200004	R05C01	3.8	0.9	4.7	1.3	5.8	1.4	11.7	20.2	99.97%
27	CH_FAM0153_S-191223-00092	203982200004	R07C01	10.9	2.3	5.9	1.5	6.5	1.6	14.7	18.1	99.96%

For sample 26, The “Bisulfite conversion 1 background green” probe is below the default value of 1. But since it is within the allowed QC metrics (maximum one Bisulfite conversion control (out of six) failed), the QC metric for this sample is considered sufficient.

Methylation classifier QC

Using the classifier, a CNS subtype would be generated based on the methylation profile relative to the CNS tumor reference set. For each methylation family and methylation class, there are associated scores, which represent the confidence of each call.

In the example below, this sample was categorized as “medulloblastoma, subclass SHH A (children and adult)”. The methylation family is medulloblastoma, which has a score of 0.9998. The methylation class is SHH A (children and adult”, which also happens to have a score of 0.9998 (family and class scores are independent values). These data suggest this tumor sample is confidently categorized to this specific medulloblastoma subtype.

Results from this assay will be reported in a qualitative manner in accordance with the following:

Classifier Family score	Category
0.8 – 1	Consistent with
0.5 – 0.79	No match, but suggestive of
0 – 0.49	Tumor classification not determined

Thus, this example would be reported as:

Methylation Class: Consistent with medulloblastoma, subclass SHH A (children and adult)

MNP Methylation Profiling Report (v11b6)

30 September, 2020

Sample Information

ID	Sentrix ID	Array type	Material type	Gender
M20-2081_S-200425-00001_Methylation_report	204086160025_R04C01	IlluminaHumanMethylationEPIC	FFPE	Male

Brain Tumor Methylation Classifier

Methylation Family Scores

Family Score	Methylation Family
0.9998	MTGF_MB_SHH
0.0001	MTGF_IDH_GLM
0.0001	MTGF_MB_G3G4
0.0001	MTGF_GBM
0.0001	EPN, PF A

Methylation Class Scores

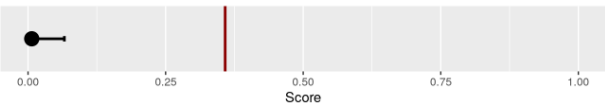
Class Score	Methylation Class
0.9998	MB, SHH CHL AD
0.0001	A IDH, HG
0.0001	MB, G3
0.0001	MB, SHH INF
0.0001	MB, G4

Methylation Class Description

The methylation class "medulloblastoma, subclass SHH A (children and adult)" is comprised of tumors diagnosed as Medulloblastoma, genetically defined, SHH-activated occurring in non-infant patients. Histologically most cases fall into the desmoplastic variant, sometimes classic and occasionally large cell/anaplastic groups. Tumors are located in the cerebellum, usually laterally. Median age is 22 years (range 3 to 51). Upstream SHH pathway alterations (i.e. PTCH1 and SMO) are relatively common. Importantly, this methylation class also includes the majority of TP53-mutated SHH tumors (often Li-Fraumeni associated), which typically occur in children (~8-16 years) and often have large cell/anaplastic morphology, with dramatic copy number alterations (chromothripsis).

In the example below, the sample is classified as unmethylated for the *MGMT* promoter.

MGMT Prediction



Status	Estimated	CI_Lower	CI_Upper	Cutoff
unmethylated	0.0067415	0.0006513	0.0660145	0.3582

see: Pierre Bady, Davide Sciuscio, Annie-Claire Diserens et al. *MGMT* methylation analysis of glioblastoma on the Infinium methylation BeadChip identifies two distinct CpG regions associated with gene silencing and outcome, yielding a prediction model for comparisons across datasets, tumor grades, and CIMP-status. Acta Neuropathologica, p.547-560, Number 4, 2012.

Concordance of Version 1 vs Version 2 of the Array Chip

In 2020, Illumina resynthesized the MethylationEPIC beadchip probes. While the resultant probe content was unchanged, the classifier algorithm was updated to maximize performance. To ensure concordance in performance, we analyzed samples that were previously run in version 1, against version 2 of the classifier. Data of the version 1 of the array chip was analyzed using classifier version 11b4, and version 2 was analyzed using 11b6.

Version 1						Version 2					
Index	Sentrix	Subtype	Family	Class	QC	Sentrix	Sentrix	Subtype	Family	Class	QC
code	Position		Score	Score		Barcode	Position		Score	Score	
360243	R03C01	low grade glioma, subclass dysembryoplastic neuroepithelial tumor	0.167	0.2084	passed	204316970049	R01C01	low grade glioma, subclass dysembryoplastic neuroepithelial tumor	0.3147	0.2986	passed
4720101	R05C01	CNS neuroblastoma with FOXR2 activation	0.11	n/a	passed	204316970049	R02C01	CNS neuroblastoma with FOXR2 activation	0.2723	n/a	passed
290012	R04C01	low grade glioma, subclass ganglioglioma	0.2077	n/a	passed	204316970049	R03C01	control tissue, hemispheric cortex	0.1856	n/a	passed
500153	R03C01	Medulloblastoma, WNT	0.9998	n/a	passed	204316970049	R04C01	Medulloblastoma, WNT	0.9996	n/a	passed
460126	R06C01	medulloblastoma, WNT	0.9993	n/a	passed	204316970049	R05C01	Medulloblastoma, WNT	0.9998	n/a	passed

In the table above, results for five samples that were analyzed with both software versions are displayed. Four of the samples had concordant subtype results. For sample “JL-17-014_H3950”, the subtype results were discordant. However, because of the low classifier score generated from both runs, the discordant result may be associated with updates related to retraining of the classifier in the newer version. Given that this sample had passed array QC for both runs, it is unlikely that this discordant was due to technical errors. Importantly, results from software versioning updates would not have impacted the overall category in which a tumor classification was reported out with family scores remaining consistently classified across all samples.

Results from this assay will be reported in a qualitative manner in accordance with the following:

Classifier Family score	Category
0.8 – 1	Consistent with
0.5 – 0.79	No match, but suggestive of
0 – 0.49	Tumor classification not determined

Assay Limitation

This assay is reliant on the [German Cancer Research Center \(DKFZ\)](#), [German Consortium for Translational Cancer Research \(DKTK\)](#), and [University Hospital Heidelberg](#) (<https://www.molecularneuropathology.org/mnp>) CNS methylation classifier software, which includes a reference set of >90 CNS tumor subtypes. For rare tumor subtypes, it may not match to any of the reference CNS tumor subtypes. Moreover, this assay is not a stand-alone test, and it should be interpreted in conjunction with other histology and molecular testing.

Validation Design/Results	To validate this assay, we processed a cohort of CNS tumor samples that had been previously run in another CLIA lab using methylation array technology and application of a classifier. The subtype results of these samples were obtained by interpersonal communication between physicians, or through clinical reports. The family and class score however are not described because they were generally unavailable with most reports being qualitative in nature.
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Sample Name	Reference Laboratory	Reference subtype
CH_FAM0087	Cincinnati	medulloblastoma, subclass group 3
CH_FAM0032	NYU	pineoblastoma group A / intracranial retinoblastoma
JL-18-036	NYU	diffuse midline glioma subclass H3 K27M mutant
JL-18-037	NYU	glioblastoma, IDH wildtype, subclass mesenchymal
CH_FAM0147	NYU	CNS neuroblastoma with FOXR2 activation
CH_FAM0161	Cincinnati	medulloblastoma, subclass group 4
CH_FAM0140	NYU	plexus tumor, subclass pediatric B
CH_FAM0204	NYU	glioblastoma, IDH wildtype, subclass MYCN
CH_FAM0216	NIH	plexus tumor, subclass pediatric A
CH_FAM0028_Z0474	DKFZ	plexus tumor, subclass pediatric B
CH_FAM0248_S-2020-001635	NIH	diffuse midline glioma H3K27 mutant
CH_FAM0238_S-2020-002381	NIH	medulloblastoma, subclass group 4
CH_FAM0251_S-2020-003556	Cincinnati	medulloblastoma, subclass group 4
CH_FAM0237_S-200814-00015	NIH	diffuse astrocytoma, MYB/MYBL1
CH_FAM0241_S-2020-001762	NIH	(anaplastic) pleomorphic xanthoastrocytoma
NABSIW-0BYJ5_CT 120720-1	NIH	low grade glioma, subependymal giant cell astrocytoma

These samples were analyzed using the methodology described in the section above.

The samples below were used to help determine the *MGMT* methylation status of CNS tumor samples.

Sample Name	Reference Laboratory	Reference <i>MGMT</i> status
CH_FAM0042	OSU	unmethylated
CH_FAM0011	Cleveland Clinic	unmethylated
JL-18-036	St. Jude	unmethylated
CH_FAM0020	St. Jude	unmethylated
CH_FAM0153	OSU	unmethylated
CH_FAM0133	OSU	methylated
CH_FAM0140	OSU	unmethylated
CH_FAM0201	U. Chicago	methylated
CH_FAM0028_Z0474	DKFZ	unmethylated
CH_FAM0248_S-2020-001635	OSU	unmethylated
CH_FAM0241_S-2020-001762	NIH	unmethylated

Accuracy

CNS tumor classification

Reference Laboratory	Reference subtype	Sentrix Barcode	Sentrix Position	Subtype from this validation	Family Score	Class Score
Cincinnati	medulloblastoma, subclass group 3	203550290012	R07C01	medulloblastoma, subclass group 3	0.9993	0.9985
NYU	pineoblastoma group A / intracranial retinoblastoma	203550290008	R05C01	pineoblastoma group A / intracranial retinoblastoma	0.9983	n/a
NYU	diffuse midline glioma subclass H3 K27M mutant	203550290008	R02C01	diffuse midline glioma subclass H3 K27M mutant	0.1622	0.1283
NYU	glioblastoma, IDH wildtype, subclass mesenchymal	203550290054	R05C01	glioblastoma, IDH wildtype, subclass mesenchymal	0.18	0.1119
NYU	CNS neuroblastoma with FOXR2 activation	203991470101	R05C01	CNS neuroblastoma with FOXR2 activation	0.1163	n/a
Cincinnati	medulloblastoma, subclass group 4	203991460142	R02C01	medulloblastoma, subclass group 4	0.7426	0.7112
NYU	plexus tumor, subclass pediatric B	203991470087	R04C01	plexus tumor, subclass pediatric B	0.2463	0.2209
NYU	glioblastoma, IDH wildtype, subclass MYCN	204229180128	R04C01	glioblastoma, IDH wildtype, subclass MYCN	0.9999	0.9993
NIH	plexus tumor, subclass pediatric A	204229180089	R04C01	plexus tumor, subclass pediatric A	0.9999	0.9998
DKFZ	plexus tumor, subclass pediatric B	204316900160	R01C01	plexus tumor, subclass pediatric B	0.9638	0.9742
335 NIH	diffuse midline glioma H3K27 mutant	204316900160	R02C01	diffuse midline glioma H3 K27M mutant	0.9999	n/a
881 NIH	medulloblastoma, subclass group 4	204316900160	R03C01	medulloblastoma, subclass group 4	0.995	0.9411
356 Cincinnati	medulloblastoma, subclass group 4	204316900160	R04C01	medulloblastoma, subclass group 4	0.9991	0.999
2015 NIH	diffuse astrocytoma, MYB/MYBL1	204316900160	R05C01	low grade glioma, MYB/MYBL1	0.9736	n/a
762 NIH	(anaplastic) pleomorphic xanthoastrocytoma	204316900160	R06C01	(anaplastic) pleomorphic xanthoastrocytoma	0.9673	n/a
>1	low grade glioma, subependymal giant cell astrocytoma	204920830048	R07C01	low grade glioma, subependymal giant cell astrocytoma	0.8589	n/a

For all 16 samples, our assay generated the subtypes that were concordant to the reference samples. Of note, all of these samples met the established array QC metrics. However, as noted in the Family Score and Class Score column, some samples had scores of <0.9 (JL-18-036, JL18-037, CH_FAM0147, CH_FAM0161, CH_FAM0140). The lower scores are likely due to the innate tumor biology that led to low confidence in tumor subtyping, however concordance in the classifier calling the same subtype between laboratories highlights consistency in performance. For CH_FAM0237, because NIH provides an integrated result that combines methylation and histology results, that may lead to the discrepancy in the final classification with our result, which was based on methylation result only.

MGMT Promoter Methylation Status

Sample Name	Reference Laboratory	Reference MGMT status	Sentrix Barcode	Sentrix Position	MGMT status	MGMT score
CH_FAM0042	OSU	unmethylated	203212860216	R05C01	Unmethylated	0.0086228
CH_FAM0011	Cleveland Clinic	unmethylated	203550290055	R04C01	Unmethylated	0.0044757
JL-18-036	St. Jude	unmethylated	203550290008	R02C01	Unmethylated	0.001354
CH_FAM0020	St. Jude	unmethylated	203550290088	R06C01	Unmethylated	0.0245136
CH_FAM0153	OSU	unmethylated	203982200004	R07C01	Unmethylated	0.1327532
CH_FAM0133	OSU	methylated	203991470087	R03C01	Methylated	0.9999139
CH_FAM0140	OSU	unmethylated	203991470087	R04C01	Unmethylated	0.0075842
CH_FAM0201	U. Chicago	methylated	204229180040	R03C01	Methylated	0.9180508
CH_FAM0028_Z0474	DKFZ	unmethylated	204316900160	R01C01	unmethylated	0.0392891
CH_FAM0248_S-2020-001635	OSU	unmethylated	204316900160	R02C01	unmethylated	0.0203913
CH_FAM0241_S-2020-001762	NIH	unmethylated	204316900160	R06C01	unmethylated	0.0234347

For all 11 samples, our assay generated *MGMT* promoter methylation results that were concordant to the reference samples.

Sensitivity and Precision

Array performance of FFPE tissues with low DNA input

The default DNA input for the methylation array recommended by Illumina is 250ng. However, for FFPE samples, the DNA quantity could be less than 250ng due to the limited amount of tissues available. Herein, we test whether we can decrease the DNA input from FFPE sample while passing array QC and maintaining the classifier score.

We use two FFPE samples: one is medulloblastoma, one is plexus tumor. Both of which have specific subclasses.

Sample Name	Sentrix Barcode	Sentrix Position	Subtype	Family Score	Class Score	QC
250ng_S-2020-006496	204920840092	R01C01	medulloblastoma, subclass SHH A (children and adult)	0.9997	0.9997	passed
200ng_S-2020-006496	204920840092	R02C01	medulloblastoma, subclass SHH A (children and adult)	0.9998	0.9998	passed
150ng_S-2020-006496	204920840092	R03C01	medulloblastoma, subclass SHH A (children and adult)	0.9998	0.9998	passed
100ng_S-2020-006496	204920840092	R04C01	medulloblastoma, subclass SHH A (children and adult)	0.9998	0.9998	passed
250ng_S-2020-006501	204920840092	R05C01	plexus tumor, subclass pediatric B	0.9996	0.9986	passed
200ng_S-2020-006501	204920840092	R06C01	plexus tumor, subclass pediatric B	0.9995	0.998	passed
150ng_S-2020-006501	204920840092	R07C01	plexus tumor, subclass pediatric B	0.9877	0.9777	passed
100ng_S-2020-006501	204920840092	R08C01	plexus tumor, subclass pediatric B	0.9995	0.9981	passed

From the table above, DNA input of 100ng will retain high family score and class score (>0.98). The array QC passed for all samples of various DNA input (down to 100ng).

Based on this data, we determine that we would accept samples with at least 100ng DNA.

Specificity

To examine the specificity, we ran non-CNS tumor samples through the CNS classifier in this assay.

Sample Name	Histology	CNS Classifier	Family	Class
CH_FAM0044	Osteosarcoma	meningioma	0.2146	n/a
CH_FAM0071	Osteosarcoma	meningioma	0.202	n/a
CH_FAM0174	Ewing Sarcoma	Ewing Sarcoma	0.9978	n/a
CH_FAM0188	Chondrosarcoma	meningioma	0.1057	n/a
CH_FAM0195	Osteosarcoma	meningioma	0.0942	n/a
CH_FAM0198	Ewing Sarcoma	Ewing Sarcoma	0.9916	n/a
CH_FAM0200	Sarcoma	Ewing Sarcoma	0.5507	n/a
CH_FAM0200	Sarcoma	Ewing Sarcoma	0.5507	n/a
CH_FAM0203	Rhabdomyosarcoma	medulloblastoma, subclass SHH A (children and adult)	0.0845	0.0567
CH_FAM0226	alveolar rhabdomyosarcoma	plexus tumor, subclass pediatric B	0.0809	0.0601
CH_FAM0234	atypical osteoblastic proliferation	hemangioblastoma	0.2628	n/a
CH_FAM0264	desmoplastic fibroma	meningioma	0.2792	n/a

In all of the non-CNS samples tested above (n=12), the results either demonstrated a low family score with no match to histology (as expected), or they matched when a non-CNS malignancy which was also included in the CNS classifier (e.g. Ewing Sarcoma). Importantly, all Family scores would have enabled the appropriate final categorization on the report with histologies lacking representation in the classifier and thus with low Family scores reported as **Tumor Classification Not Determined**, sarcomas with a moderate score reported as **No Match but Suggestive of Ewing Sarcoma** (based on the closest available match), and Ewing Sarcoma confidently classified as **Consistent with Ewing Sarcoma**.

Methylation class Ewing sarcoma description as included in the CNS classifier:

The methylation class "Ewing sarcoma" typically displays a small-cell phenotype with variable, embryonal histology. The cases comprising this reference class were located spinally (extradural) or around the skull base, with several lung metastases also included. Median age is 18.5 years (range 8 to 36). Molecularly, these tumors are characterized by typical EWSR1 fusions. Among other copy number changes, gain of chromosome 8 is particularly frequent (>50% of cases).

Classifier Family score	Category
0.8 – 1	Consistent with
0.5 – 0.79	No match, but suggestive of
0 – 0.49	Tumor classification not determined

From these data, we determine that **the analytical specificity is 100%**.

Array performance of tumors with low tumor cellularity

Frequently, the CNS tumor samples submitted do not have 100% tumor cellularity. We test the lower limit of tumor cellularity while passing QC and achieving high family and class score.

	Sample Name	Sentrix Barcode	Sentrix Position	Subtype	Family Score	Class Score	QC
Medulloblastoma	100% FAM0079 / 0% JLE-002	204229180039	R01C01	Medulloblastoma, subclass group 4	0.9999	0.9999	Passed
	80% FAM0079 / 20% JLE-002	204229180039	R02C01	Medulloblastoma, subclass group 4	0.9704	0.9535	Passed
	70% FAM0079 / 30% JLE-002	204229180039	R03C01	Medulloblastoma, subclass group 4	0.7755	0.6845	Passed
	60% FAM0079 / 40% JLE-002	204229180039	R04C01	Medulloblastoma, subclass group 4	0.4111	0.3241	Passed
	50% FAM0079 / 50% JLE-002	204229180039	R05C01	Medulloblastoma, subclass group 4	0.1443	0.0954	Passed
ATRT	100% FAM0170 / 0% JLE-002	204229180088	R01C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9999	0.9999	Passed
	80% FAM0170 / 20% JLE-002	204229180088	R02C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9999	0.9998	Passed
	70% FAM0170 / 30% JLE-002	204229180088	R03C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9995	0.9991	Passed
	60% FAM0170 / 40% JLE-002	204229180088	R04C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9964	0.9925	Passed
	50% FAM0170 / 50% JLE-002	204229180088	R05C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9354	0.9739	Passed

As demonstrated in the table above, we tested two tumor samples with 100% tumor cellularity. To test the lower limit, we diluted the tumors with DNA from normal cerebral cortex, which would mimic the normal brain methylation profile.

We diluted the tumor samples to 80%, 70%, 60%, and 50%. The array QC metrics passed for all samples. We found that the ATRT sample retained the family and class score down to 50% with highly confident scores, while the family and class scores for the medulloblastoma sample fell below 0.90 when the tumor cellularity drops down to 70%.

Based on this data, we determined that we would accept samples with tumor cellularity of 60% or higher.

Inter-technologist reproducibility

Tech 1					Tech 2				
Sentrix Position	Subtype	Family Score	Class Score	QC	Sentrix Barcode	Sentrix Position	Subtype	Family Score	Class Score
R01C01	low grade glioma, subclass dysembryoplastic neuroepithelial tumor	0.3147	0.2586	passed	204316900130	R01C01	low grade glioma, subclass dysembryoplastic neuroepithelial tumor	0.3419	0.3419
R02C01	CNS neuroblastoma with FOXR2 activation	0.2723	n/a	passed	204316900130	R02C01	CNS neuroblastoma with FOXR2 activation	0.2816	n/a
R03C01	control tissue, hemispheric cortex	0.1856	n/a	passed	204316900130	R03C01	control tissue, hypothalamus	0.3382	n/a
R04C01	Medulloblastoma, WNT	0.9996	n/a	passed	204316900130	R04C01	Medulloblastoma, WNT	0.9999	n/a
R05C01	Medulloblastoma, WNT	0.9998	n/a	passed	204316900130	R05C01	Medulloblastoma, WNT	0.9999	n/a

Two different technologists independently ran five different samples on separate runs. The results for all samples from both runs were concordant with no change in final categorization based on Family score and consistency in subtypes (with one sample of control tissue demonstrating a slight difference in location according to the classifier).

Inter-run and intra-technologist reproducibility

	Sentrix Barcode	Sentrix Position	Subtype	Family Score	Class Score	QC	Sentrix Barcode	Sentrix Position	Subtype	Family Score	Class Score	QC
2	204229180039	R01C01	Medulloblastoma, subclass group 4	0.9999	0.9999	Passed	204316900106	R01C01	Medulloblastoma, subclass group 4	0.9999	0.9999	Passed
2	204229180039	R02C01	Medulloblastoma, subclass group 4	0.9704	0.9535	Passed	204316900106	R02C01	Medulloblastoma, subclass group 4	0.9702	0.9543	Passed
2	204229180039	R03C01	Medulloblastoma, subclass group 4	0.7755	0.6845	Passed	204316900106	R03C01	Medulloblastoma, subclass group 4	0.7767	0.7107	Passed
2	204229180039	R04C01	Medulloblastoma, subclass group 4	0.1111	0.3241	Passed	204316900106	R04C01	Medulloblastoma, subclass group 4	0.3805	0.2887	Passed
2	204229180039	R05C01	Medulloblastoma, subclass group 4	0.1443	0.0854	Passed	204316900106	R05C01	Medulloblastoma, subclass group 4	0.1435	0.0949	Passed
2	204229180088	R01C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9999	0.9999	Passed	204316900151	R01C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9998	0.9997	Passed
2	204229180088	R02C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9999	0.9998	Passed	204316900151	R02C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9996	0.9994	Passed
2	204229180088	R03C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9995	0.9991	Passed	204316900151	R03C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9989	0.9982	Passed
2	204229180088	R04C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9964	0.9925	Passed	204316900151	R04C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9927	0.9855	Passed
2	204229180088	R05C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9354	0.9739	Passed	204316900151	R05C01	atypical teratoid/rhabdoid tumor, subclass TYR	0.9509	0.8811	Passed

10 samples were analyzed on two different runs by the same technologist. The results for all samples from both runs were concordant.

Intra-run reproducibility

	Sample Name	Sentrix Barcode	Sentrix Position	Subtype	Family Score	Class Score	QC
	Z0604	203212860216	R05C01	medulloblastoma, subclass group 4	0.9997	0.9997	passed
		203212860216	R06C01	medulloblastoma, subclass group 4	0.9997	0.9997	passed
	Z0603	203212860216	R07C01	control tissue, cerebellar hemisphere	0.9605	0.9605	passed
		203212860216	R08C01	control tissue, cerebellar hemisphere	0.9583	0.9583	passed
	500 ng Zymo Human Methylated DNA	203212860185	R01C01	lymphoma	0.7392	0.7392	passed
		203212860185	R02C01	lymphoma	0.7363	0.7363	passed
	500 ng Zymo Human Non-Methylated DNA	203212860185	R03C01	plexus tumor, subclass pediatric B	0.3937	0.3818	passed
		203212860185	R04C01	plexus tumor, subclass pediatric B	0.3977	0.3857	passed
	250 ng Zymo Human Methylated DNA	203212860185	R05C01	lymphoma	0.746	0.746	passed
		203212860185	R06C01	lymphoma	0.7521	0.7521	passed
		203212860185	R07C01	plexus tumor, subclass pediatric B	0.3938	0.3818	passed
	250 ng Zymo Human Non-Methylated DNA	203212860185	R08C01	plexus tumor, subclass pediatric B	0.3915	0.3798	passed
	250ng JL-17-032 H6830	203550290025	R03C01	plexus tumor, subclass pediatric B	0.9997	0.9977	passed
	500ng	203550290025	R04C01	plexus tumor, subclass pediatric B	0.9997	0.998	passed
	250ng JL-17-032 H6831	203550290025	R05C01	plexus tumor, subclass pediatric B	0.9957	0.9903	passed
	500ng	203550290025	R06C01	plexus tumor, subclass pediatric B	0.9964	0.9917	passed

8 samples, including tumor and control samples, were analyzed in duplicates in the same run. Two samples, H6830 and H6831, were duplicate using two different DNA input. The results for all duplicates were concordant. The Zymo Human Methylated and Non-methylated DNA are sets of controls that were used to assess the efficiency of bisulfite conversion. The classification result of lymphoma is likely not significant.

Supplemental Data for New York State Validation

Additional experiments were performed for triplicates for inter-and intra-run reproducibility for the New York State approval for oncology testing.

	Inter-run reproducibility										
	Intra-run reproducibility					Intra-run reproducibility			Intra-run reproducibility		
Pathology tumor cellularity	Run 58	Run 59	Run 60			Run 61			Run 62		
75%	GBM, MD	GBM, MD	GBM, MD	GBM, MD	GBM, MD						
95%	MB, SHH, CHL, AD	MB, SHH, CHL, AD	MB, SHH, CHL, AD	MB, SHH, CHL, AD	MB, SHH, CHL, AD						
75%	ETMR	ETMR				ETMR	ETMR	ETMR			
100%	LGG, PA, PF	LGG, PA, PF				LGG, PA, PF	LGG, PA, PF	LGG, PA, PF			
90%	MB, G4	MB, G4							MB, G4	MB, G4	MB, G4
80%	ATRT, TYR	ATRT, TYR							ATRT, TYR	ATRT, TYR	ATRT, TYR
>95%	PLEX, PED A	PLEX, PED A	PLEX, PED A			PLEX, PED A			PLEX, PED A		
0%	CONTR, REACT	CONTR, REACT	CONTR, REACT			CONTR, REACT			CONTR, REACT		

GBM, MD = Glioblastoma, IDH wildtype, subclass midline
MB, SHH CHL AD = medulloblastoma, subclass SHH A (children and adult)
ETMR = embryonal tumor with multilayered rosettes
LGG, PA PF = low grade glioma, subclass posterior fossa pilocytic astrocytoma
MB, G4 = medulloblastoma, subclass group 4
ATRT, TYR = atypical teratoid/rhabdoid tumor, subclass TYR
PLEX, PED A = Plexus tumor, subclass pediatric A

	CONTR, REACT = Control tissue, reactive tumor microenvironment Six samples were using for the reproducibility calculation. Blue indicates data used for inter-run reproducibility. Grey indicates data used for intra-run reproducibility. Based on these data, the reproducibility is 100% for both intra-run and inter-run reproducibility with Family and Class descriptors matching for all samples as displayed in the table above. In summary, this validation serves to document that the CNS Tumor Classification by Methylation Array assay demonstrates robust and reproducible performance acceptable for implementation in the IGM Clinical Laboratory.
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APPENDIX E Medication Diaries
PBTC-053
Phase I & II: Patient's Diary for CX-4945 (capsule)

Today's date:	Agent: CX-4945
PBTC accession #:	Patient's Initials:
Assigned Dose:	# Capsules:

INSTRUCTIONS TO THE PATIENT:

- Complete one form for each cycle of treatment.
- CX-4945 should be taken twice each day on an empty stomach either 1 hour before or 2 hours after meals.
- Patients should take anti-emetic medication prior to taking the CX-4945 capsules.
- Record the time the anti-emetic medication was taken.
- CX-4945 capsules should be taken whole with water only.
- Patients who vomit a dose of CX-4945 should NOT be re-dosed, and appropriate anti-emetic therapy should be implemented prior to the next scheduled dose.
- Record the date, the number of CX-4945 capsules that you took, and when you took them.
- Patients participating in the pharmacokinetic (PK) monitoring studies should hold the second dose on Day 1 and the second dose on Day 28 to complete the PK sample collections.
- If you have any comments or notice any side effects, please record them in the Comments column.
- Please bring this form, any unused medication and your bottles of CX-4945 when you return for each appointment.

Course #		Pre-Med Dose Time	AM dose Time	AM: #CX-4945 200mg Capsules	Pre-Med Dose Time	PM Dose Time	PM: # CX-4945 200mg Capsules	Comments
Start date								
End date								
	Date							
Day 1								
Day 2								
Day 3								
Day 4								
Day 5								
Day 6								
Day 7								
Day 8								
Day 9								
Day 10								

Day 11								
Day 12								
Day 13								
Day 14								
Day 15								
Day 16								
Day 17								
Day 18								
Day 19								
Day 20								
Day 21								
Day 22								
Day 23								
Day 24								
Day 25								
Day 26								
Day 27								
Day 28								

Patient/Guardian's signature: _____

Date: _____

PBTC-053
Surgical Study: Patient's Diary for CX-4945 (capsules)

Today's date:	Agent: CX-4945
PBTC accession #:	Patient's Initials:
Assigned Dose:	# Capsules:

INSTRUCTIONS TO THE PATIENT:

- Complete one form for each cycle of treatment.
- CX-4945 should be taken twice each day on an empty stomach either 1 hour before or 2 hours after meals.
- Patients should take anti-emetic medication prior to taking the CX-4945 capsules.
- Record the time the anti-emetic medication was taken.
- CX-4945 capsules should be taken whole with water only.
- Patients who vomit a dose of CX-4945 should NOT be re-dosed, and appropriate anti-emetic therapy should be implemented prior to the next scheduled dose.
- Record the date, the number of capsules that you took, and when you took them.
- If you have any comments or notice any side effects, please record them in the Comments column.
- Please bring this form, any unused medication and your bottles of CX-4945 when you return for each appointment.

Course #		Pre-Med Dose Time	AM dose Time	AM: #CX-4945 200mg Capsules	Pre-Med Dose Time	PM Dose Time	PM: # CX-4945 200mg Capsules	Comments
Start date								
End date								
	Date							
Day -7								
Day -6								
Day -5								
Day -4								
Day -3								
Day -2								
Day -1								
Day 0	No study drug should be taken on the day of surgery							

Patient/Guardian's signature: _____

Date: _____