Sorafenib inhibits signal transducer and activator of transcription 3 signaling associated with growth arrest and apoptosis of medulloblastomas

Fan Yang,[1] Timothy E. Van Meter,[4] Ralf Buettner,[1] Michael Hedvat,[1] Wei Liang,[1] Claudia M. Kowolik,[1] Nilesh Mepani,[4] Janni Mirosevich,[1] Sangkil Nam,[1] Mike Y. Chen,[2] Gary Tye,[4] Mark Kirschbaum,[3] and Richard Jove[1]

[1]Molecular Medicine, [2]Neurosurgery, and [3]Hematology, Beckman Research Institute, City of Hope National Medical Center, Duarte, California and [4]Neurosurgery, Virginia Commonwealth University, Richmond, Virginia

Abstract

Medulloblastomas are the most frequent malignant brain tumors in children. Sorafenib (Nexavar, BAY43-9006), a multikinase inhibitor, blocks cell proliferation and induces apoptosis in a variety of tumor cells. Sorafenib inhibited proliferation and induced apoptosis in two established cell lines (Daoy and D283) and a primary culture (VC312) of human medulloblastomas. In addition, sorafenib inhibited phosphorylation of signal transducer and activator of transcription 3 (STAT3) in both cell lines and primary tumor cells. The inhibition of phosphorylated STAT3 (Tyr^{705}) occurs in a dose- and time-dependent manner. In contrast, AKT (protein kinase B) was only decreased in D283 and VC312 medulloblastoma cells and mitogen-activated protein kinases (extracellular signal-regulated kinase 1/2) were not inhibited by sorafenib in these cells. Both D-type cyclins (D1, D2, and D3) and E-type cyclin were down-regulated by sorafenib. Also, expression of the antiapoptotic protein Mcl-1, a member of the Bcl-2 family, was decreased and correlated with apoptosis induced by sorafenib. Finally, sorafenib suppressed the growth of human medulloblastoma cells in a mouse xenograft model. Together, our data show that sorafenib blocks STAT3 signaling as well as expression of cell cycle and apoptosis regulatory proteins, associated with inhibition of cell proliferation and induction of apoptosis in medulloblastomas. These findings provide a rationale for treatment of pediatric medulloblastomas with sorafenib. [Mol Cancer Ther 2008;7(11):3519–26]

Introduction

Primitive neuroectodermal tumors are a highly heterogeneous group of tumors arising mainly in children up to 9 years of age (1). Cerebellar primitive neuroectodermal tumors, known as medulloblastoma, are the most prevalent malignancy of the central nervous system in childhood. The etiology of medulloblastomas is still unclear, although several signaling pathways that control cell proliferation are thought to be involved in disease progression. The Sonic hedgehog and the Wingless pathways have been linked to the development of medulloblastomas (2–6). In addition, AKT (protein kinase B) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) may contribute to the progression of the tumor in certain cases (7).

The activity of signal transducer and activator of transcription (STAT) proteins, particularly STAT3, is frequently elevated in a variety of solid tumors and hematologic malignancies (8). STAT3 proteins have dual roles as cytoplasmic signaling proteins and nuclear transcription factors that activate a diverse set of genes, including some that implicated in malignant progression (9, 10). STAT3 is found to be constitutively activated in medulloblastomas (11) and the level of STAT3 activation in medulloblastomas exceeds that of all other brain tumors examined, including glioblastomas, ependymomas, and astrocytomas (12). Therefore, the formation and maintenance of medulloblastomas may be regulated in part by STAT3.

Sorafenib (Nexavar, BAY43-9006) is an oral multikinase inhibitor that was originally developed based on its inhibitory effect on Raf and receptor tyrosine kinase signaling (13). Deregulation of the Raf-MEK-MAPK signaling pathway is associated with development of solid tumors (14–16). Recent findings showed that sorafenib inhibited tumor growth and angiogenesis, and induced apoptosis, through either Raf-MEK-MAPK-dependent or Raf-MEK-MAPK-independent pathways, depending on the type of tumors being investigated (17–19). Evaluation of sorafenib from phase I and II clinical trials on several types of advanced solid tumors showed favorable tolerability and promising clinical antitumor activity (20–22).

Our present results show that sorafenib inhibits proliferation and induces apoptosis in two established human cell lines (D283 and Daoy) and a primary culture (VC312) of human medulloblastomas. Sorafenib also inhibits the *in vivo* growth of human medulloblastoma cells in nude

Received 2/13/08; revised 6/30/08; accepted 7/15/08.

Grant support: Sunshine Project of the Pediatric Cancer Foundation and National Cancer Institute grant CA1155674 (R. Jove).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Richard Jove, Molecular Medicine, Beckman Research Institute, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010. Phone: 626-301-8179; Fax: 626-256-8708. E-mail: rjove@coh.org

doi:10.1158/1535-7163.MCT-08-0138

mice. The biological effects of sorafenib on medulloblastomas are associated with inhibition of STAT3 signaling as well as down-regulation of cyclin D/E and Mcl-1 proteins. These findings suggest that sorafenib may be effective for the treatment of pediatric medulloblastoma tumors through inhibition of STAT3 signaling.

Materials and Methods

Reagents and Antibodies

Sorafenib was kindly provided by Onyx and Bayer Pharmaceuticals. Anti-cyclin D1 and D3 were obtained from Calbiochem. Anti-cyclin E was obtained from BD Biosciences. Anti-cyclin D2 and anti-Mcl-1 were obtained from Santa Cruz. Horseradish peroxidase-labeled anti-mouse and anti-rabbit secondary antibodies were from GE Healthcare. All other antibodies were obtained from Cell Signaling.

Cell Culture

Two human meduloblastoma cell lines, D283 (D283med) and Daoy, were from the American Type Culture Collection. All cells were maintained in Eagle's MEM with L-glutamine supplemented with 10% fetal bovine serum, and 1% antibiotic-antimycotic. The primary culture (VC312) of medulloblastoma was derived from a tumor of a 4-year-old male patient treated at the Virginia Commonwealth University Health System's Medical College of Virginia Hospital under an institutional review board-approved protocol. Briefly, samples of the tumor were first obtained to allow full neuropathologic evaluation and diagnosis as required for the clinical management of the patient's disease. The site of origin of all the tumor samples was cerebellum. The sterile dissection of tumor biopsy was dissociated and plated in six-well tissue culture plates and expanded in DMEM/F-12 supplemented with 1% N-2 supplement (Invitrogen), 5% fetal bovine serum, 20 ng/mL recombinant human epidermal growth factor, and 10 ng/mL recombinant human basic fibroblast growth factor (Becton Dickinson). VC312 cells were subsequently maintained in DMEM (with L-glutamine) supplemented with 10% fetal bovine serum and used at low passage number (below passage 20 for all studies).

Proliferation Assay

Cell proliferation assays were done with CellTiter 96 Aqueous One Solution Cell proliferation Assay (Promega), which contains 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). Each well of 96-well plates was seeded with 5,000 cells in culture medium with 1% fetal bovine serum. After overnight culture, the cells were treated with different concentrations of sorafenib and controls were treated with vehicle (DMSO). After 24 or 48 h treatment, MTS was added to the cells according to the supplier's protocol and absorbance was measured at 490 nm using an automated ELISA plate reader.

Apoptosis Assay

Daoy, D283, or VC312 cells (2×10^5) were seeded in 60 mm culture dishes in culture medium with 1% fetal bovine serum. The following day, the cells were treated with indicated concentrations of sorafenib for a 24 or 48 h period. After treatment, all cells including both floating and attached cells were collected, and the apoptotic cells were detected by Annexin V-FITC Apoptosis Detection Kit (BD Biosciences). The cells were stained with Annexin V-FITC and propidium iodide according to the supplier's instructions. Viable and dead cells were detected by flow cytometry in the Analytical Cytometry Core at City of Hope National Medical Center.

Immunoblotting Analysis

Total proteins (20 μg) were resolved in 4% to 15% gradient Tris-HCl gels (Bio-Rad). After gel electrophoresis, the proteins were transferred to Hybond-C membranes (Amersham). The membranes were blocked for 1 h at room temperature in 10% nonfat dry milk in 1× PBS with 0.1% Tween 20 followed by an overnight incubation at 4°C with primary antibodies in 1× PBS with 0.1% Tween 20 with 2% nonfat dry milk. The membranes were then incubated with horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature. Immunoreactivity was detected with SuperSignal West Pico substrate (Pierce).

Cell Cycle Analysis

Daoy cells were plated in 100 mm culture dishes at 5×10^5 per dish. After 24 h treatment with sorafenib, cells were harvested by trypsinization and washed with PBS. Cells were fixed in ice-cold 75% ethanol, washed, and resuspended in 1 mL PBS with 50 μg propidium iodide and 0.25 mg RNase A. Samples were assayed by Flow Cytometry.

Transfection of STAT3 Small Interfering RNA

The 27-mer dicer substrate small interfering RNA (siRNA) against STAT3 was synthesized by IDT. Cells were transfected with 50 nmol/L STAT3 siRNA using LipofectAMINE RNAiMAX (Invitrogen) according to the supplier's protocol. Control cells were transfected with negative control siRNA#1 (Ambion). After 24 h transfection, cells were seeded in 96-well plates for proliferation assays.

Tumor Xenografts

Mouse xenograft studies were done in 4- to 6-week-old nude mice obtained from National Cancer Institute-Charles River Laboratories. The mice were held in a pathogen-free animal facility at City of Hope Medical Center and were fed standard rodent chow and water *ad libitum*. All procedures followed the NIH Guidelines for the Care and Use of Laboratory Animals. Tumors were generated by harvesting Daoy cells from mid-log-phase cultures. Cells were pelleted and resuspended in a 50% mixture of Matrigel (BD Biosciences) in Eagle's MEM to 2.5×10^7/mL. This cell suspension (0.2 mL) was injected s.c. in the right flank of each mouse. Sorafenib treatment was started at 10 days after injection of Daoy cells. Animals with palpably established tumors of at least 65 mm^3 were designated to treatment groups. Treatment with 100 μL of 10 μmol/L sorafenib in 1× PBS was administered by intratumoral injection three times a week for 5 weeks. Mice in the control group were injected with the same amount of PBS with

vehicle. Tumors were measured every 3 to 4 days with Vernier calipers, and tumor volumes were calculated by the formula: $\pi / 6 \times$ (larger diameter) $\times$ (small diameter)^2.

Statistics

Student's t test was used to evaluate statistical significance of differences between two groups and $P < 0.05$ was considered statistically significant.

Results

Sorafenib Inhibits Proliferation and Induces Apoptosis in D283 and Daoy Cells

To characterize the effects of sorafenib on cell proliferation in medulloblastomas, we did dose-response and time-course studies in D283 and Daoy cells (Fig. 1A and B). Cells were treated with increasing concentrations of sorafenib (2.5, 5, and 10 μmol/L) for 24 and 48 h or with the vehicle (DMSO) only as control. Because previous studies suggest that sorafenib binds to serum proteins (13), these assays were done in 1% serum to reduce the effect of serum. Sorafenib markedly inhibited proliferation of both D283 and Daoy cells in a dose- and time-dependent manner. We next investigated whether sorafenib could induce apoptosis in D283 and Daoy cells. After treatment with increasing concentrations of sorafenib (2.5, 5, 10, and 20 μmol/L) for 48 h, cells were analyzed by Annexin V/propidium iodide staining and flow cytometry. Apoptotic cells shown in Fig. 1C and D were Annexin V and propidium iodide double-positive. Results show that D283 cells were more sensitive to sorafenib than Daoy cells.

Sorafenib Inhibits Phosphorylation of STAT3 at Tyr^{705} in D283 and Daoy Cells

We investigated the levels of total and phosphorylated STAT3, AKT, and MAPK (p42/p44) proteins in D283 and Daoy cells after sorafenib treatment. Total protein levels of STAT3, AKT, and MAPK were not significantly changed after 4 or 24 h sorafenib treatment (Fig. 2A and B). By contrast, phosphorylation of STAT3 at Tyr^{705} was substantially reduced at both an early time point (4 h) and a late time point (24 h) in D283 and Daoy cells following sorafenib treatment (Fig. 2A and B). The phosphorylation of STAT3 at Tyr^{705} was inhibited in a dose- and time-dependent manner. Phosphorylated AKT was only decreased in D283 cells after 24 h treatment, and MAPK (p42/p44) was not reduced by sorafenib treatment. Phosphorylation of STAT3 at Tyr^{705} is essential for STAT3 activation (23). These results indicate that inhibition of STAT3 signaling is an early response to sorafenib treatment and that STAT3 inhibition is a common response to sorafenib in both D283 and Daoy cells.

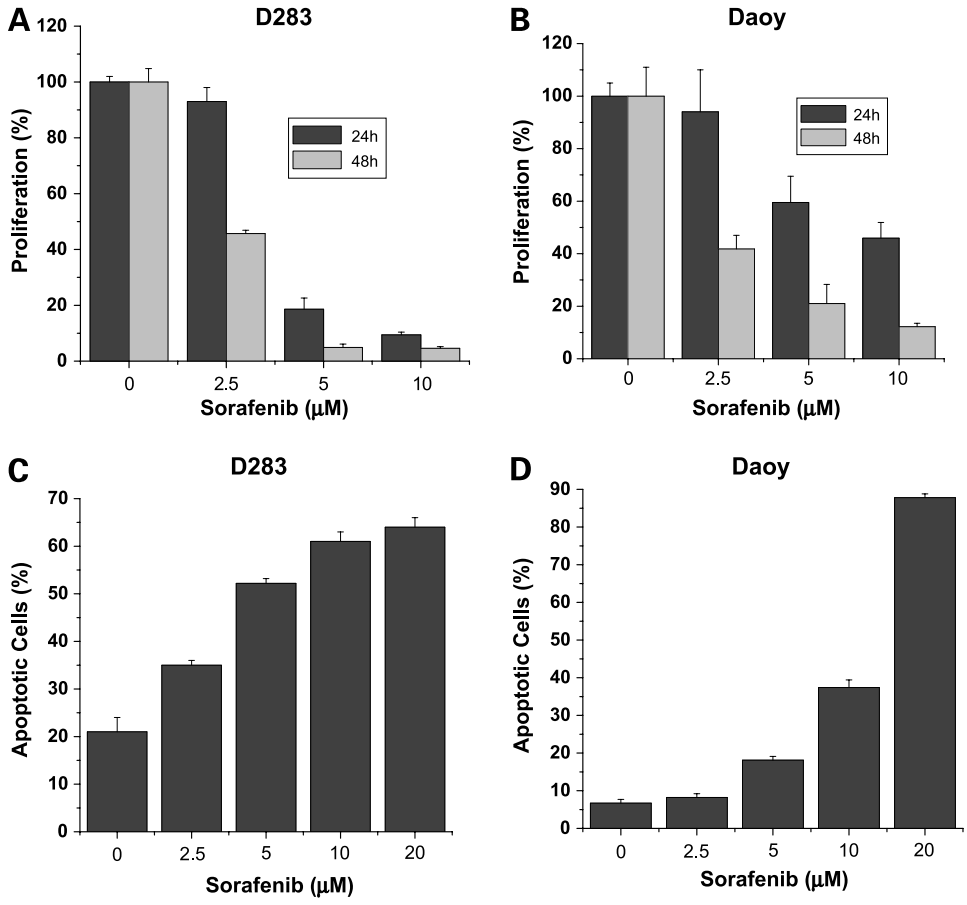

Figure 1. Sorafenib inhibits proliferation and survival of D283 and Daoy cells. **A** and **B,** effects of sorafenib on proliferation of D283 and Daoy cells. Cells were treated with 0, 2.5, 5, or 10 μmol/L sorafenib for 24 and 48 h. Cell proliferation was evaluated by MTS assay. Sorafenib induced apoptosis (**C**) and (**D**) in D283 and Daoy cells after 48 h treatment. Apoptotic cells represented propidium iodide and Annexin V-FITC double-positive cells as determined by flow cytometry. Each experiment was done in triplicate or duplicate and repeated twice independently. *Columns,* mean; *bars,* SD.

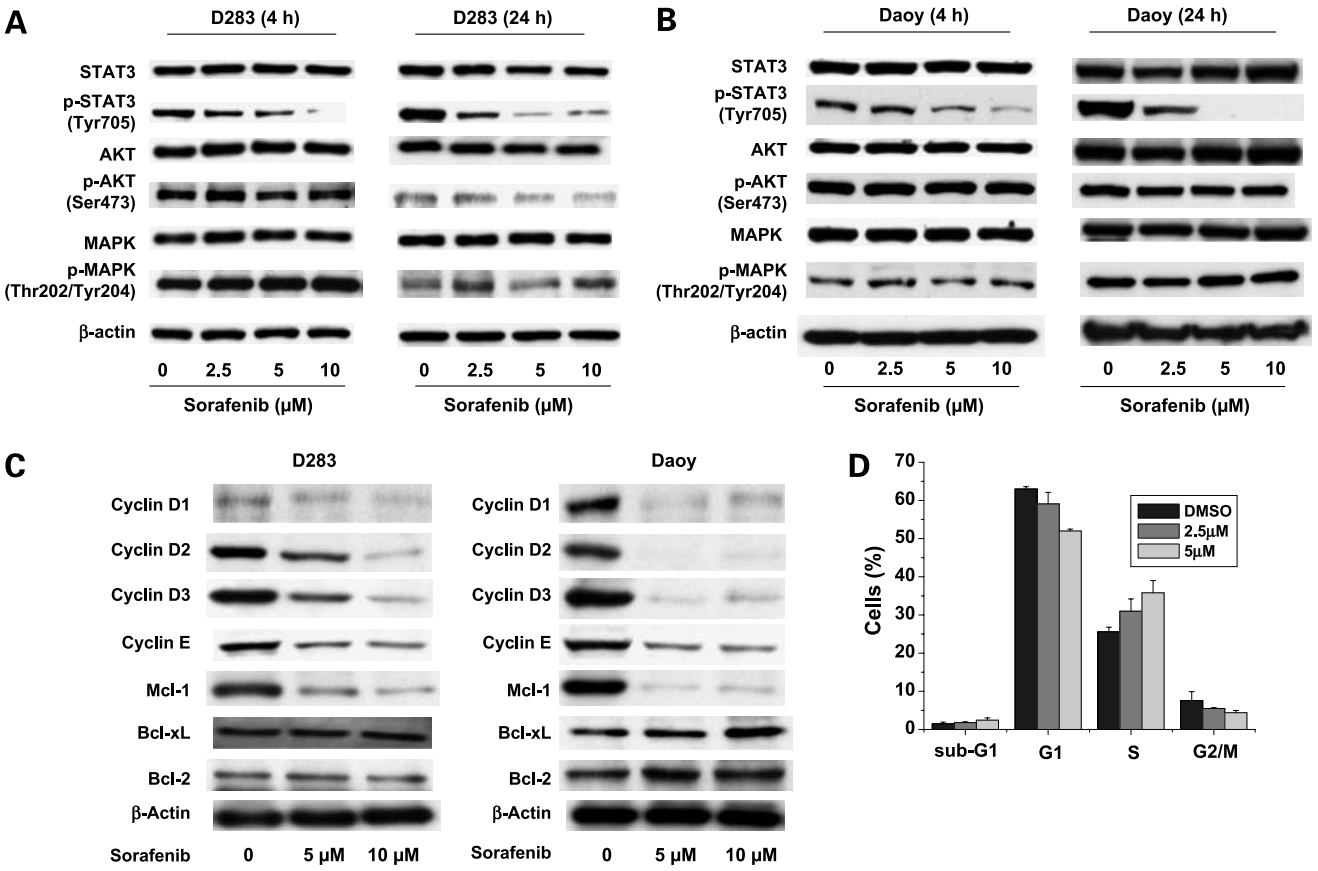

Figure 2. Effects of sorafenib on signaling pathways and proteins that control cell cycle or apoptosis. Total proteins were isolated from D283 and Daoy cells incubated with 2.5, 5, or 10 μmol/L sorafenib for 4 or 24 h. Immunoblotting in **A** and **B** was done with antibodies to total or phosphorylated (*P*) STAT3, AKT, and MAPK (p44/42) using 20 μg total proteins from D283 and Daoy cells. Anti-β-actin monoclonal antibody was used as a loading control. Western blots in **C** show the effects of sorafenib on expression of cyclins D1, D2, D3, and E as well as Mcl-1, Bcl-2, and Bcl-xL in D283 and Daoy cells after 24 h treatment. **D,** cell cycle distribution of Daoy cells following sorafenib treatment. Cells were treated with 0, 2.5, and 5 μmol/L sorafenib for 24 h and stained with propidium iodide. Cell cycle distribution was assessed by flow cytometry.

Sorafenib Inhibits Expression of Cyclin D1/D2/D3 and E in D283 and Daoy Cells

Because sorafenib significantly inhibits proliferation of D283 and Daoy cells (Fig. 1), we investigated the effect of sorafenib on key cell-cycle regulators, including D- and E-type cyclins. Immunoblot analyses were done to determine the expression of cyclin D1/D2/D3 and E in D283 and Daoy cells after 24 h sorafenib treatment. Figure 2C showed that sorafenib decreased the expression of cyclin D1/D2/D3 and E in a dose-dependent manner. Lower levels of cyclin D1 protein expression in D283 cells correlated with lower levels of cyclin D1 mRNA in these cells as confirmed by real-time reverse transcription-PCR (data not shown). We also analyzed the effect of sorafenib on cell cycle distribution in Daoy cells by fluorescence-activated cell sorting with propidium iodide staining. Figure 2D shows a decrease of cells in G_1 phase and an increase of cells arrested in S phase with increasing concentrations of sorafenib. Reducing the expression of cyclin D and E is consistent with the S-phase arrest. The S-phase arrest of Daoy cells by sorafenib is similar to an earlier report in hepatocellular carcinoma (17).

Sorafenib Inhibits Mcl-1 Expression in D283 and Daoy Cells

Bcl-2 family proteins have a key role in survival of normal and tumor cells (24). The expression of three antiapoptotic proteins in this family, Mcl-1, Bcl-xL, and Bcl-2, was investigated after sorafenib treatment. Although Bcl-xL and Bcl-2 were not decreased, Mcl-1 was decreased in both D283 and Daoy cells after 24 h of sorafenib treatment (Fig. 2C). These results are consistent with the induction of apoptosis by sorafenib (Fig. 1C and D) and implicate Mcl-1 in this response.

STAT3 Proteins Are Constitutively Activated in Primary Pediatric Medulloblastomas

Because STAT3 proteins have an important role in the progression and maintenance of many malignant tumors, we investigated the expression of STAT3 proteins in pediatric medulloblastomas. Six human medulloblastoma biopsies (MB1-MB6) were examined by Western blot

analysis with specific STAT3 antibodies. Normal rat brains including embryonic brain day 15, postnatal day 4 cerebellum, and 8-week adult cerebellum were used as controls. As shown in Fig. 3A, STAT3 protein was expressed in both rat normal brains and human tumor samples. STAT3 phosphorylation at Ser^{727} was only detected in tumor samples. Importantly, STAT3 phosphorylation at Tyr^{705} was observed in all six tumor biopsies and early developmental time points in rat brains but not in adult rat brains.

To further assess the role of STAT3 proteins in tumor cells from medulloblastoma, we employed STAT3 siRNA to inhibit the expression of STAT3 in a primary culture (VC312), which was derived from the MB3 biopsy (Fig. 3A). After transfection with STAT3 siRNA, expression of STAT3 proteins in VC312 cells was partially inhibited (Fig. 3B), and the proliferation of VC312 cells was partially decreased (Fig. 3C).

Sorafenib Inhibits Cell Proliferation and Survival in a Primary Culture of Human Medulloblastoma

To evaluate whether sorafenib has the same effect on primary cultures as on established cell lines, adherent VC312 cells were treated with sorafenib in the same manner as D283 and Daoy cells. Proliferation of VC312 cells was inhibited by sorafenib in a dose- and time-dependent manner (Fig. 4A). Sorafenib also induced apoptosis of VC312 cells in a dose-dependent manner after 48 h treatment (Fig. 4B). A lower concentration (2.5 μmol/L) of sorafenib effectively inhibited the proliferation of VC312 cells, but it could not induce apoptosis. These results indicate that relatively higher concentrations are required to induce cell death than to inhibit proliferation. This inhibitory effect on the proliferation and survival of VC312 cells is similar to that observed for D283 and Daoy cells (Fig. 1).

Sorafenib Inhibits Phosphorylation of STAT3 in a Primary Medulloblastoma Culture

We next examined signaling by STAT3, AKT, and MAPK (p42/p44) proteins after sorafenib treatment in VC312 cells. Total protein levels of STAT3, AKT, and MAPK were not changed (Fig. 4C). The levels of phosphorylated STAT3 at Tyr^{705} proteins were significantly decreased at both an early time point (4 h) and a late time point (24 h) in a dose- and time-dependent manner (Fig. 4C). Phosphorylated AKT was found to be diminished by sorafenib treatment, but inhibition of phosphorylated STAT3 was greater than that of phosphorylated AKT, especially at 4 h. Interestingly, although high levels of phosphorylated MAPK (p44/42) were present in VC312 cells, phosphorylation of MAPK (p42/p44) was not decreased by sorafenib (Fig. 4C). These results indicate that the effect of sorafenib in VC312 cells is independent of the MAPK pathway.

Sorafenib Inhibits Expression of Cyclin D1/D2/D3 and E and Mcl-1 in VC312 Cells

We also investigated the effects of sorafenib on cell cycle regulators, cyclin D and E, and antiapoptotic genes, Mcl-1,

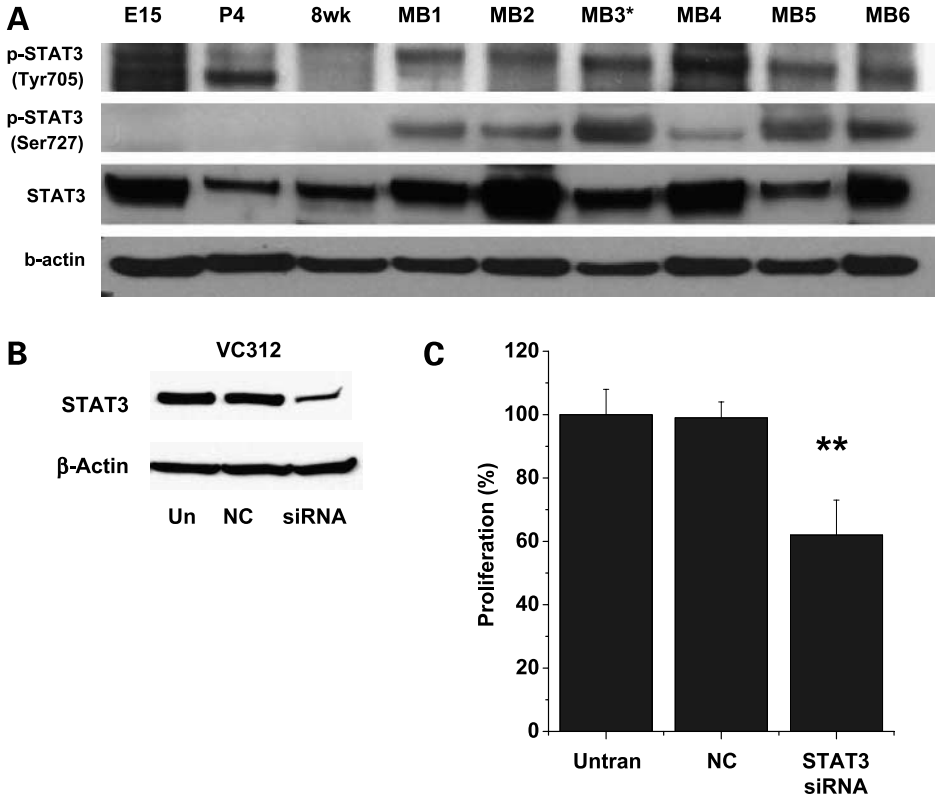

Figure 3. Expression of STAT3 in human medulloblastoma biopsies and effect of STAT3 siRNA on proliferation in a primary culture. **A,** Western blots show total STAT3 and phosphorylated STAT3 at Tyr^{705} or Ser^{727} levels in tissue protein lysates prepared from six human medulloblastoma biopsies. Different developmental time points in the rat cerebellum served as normal controls. Detection of β-actin was included as a loading control. *E15,* embryonic rat brain day 15; *P4,* postnatal day 4 rat cerebellum; *8wk,* cerebellum from 8-week-old adult rat; *MB,* medulloblastoma. *Asterisk,* VC312 cultures derived from the MB3 primary tumor. **B,** immunoblots show that expression of total STAT3 was reduced by STAT3 siRNA in a primary culture (VC312). After 24 h transfection, cells were seeded in 96-well plates and incubated another 24 h culture for proliferation assay. **C,** transfection of STAT3 siRNA into VC312 cells partially inhibited their proliferation. *Untran,* untransfected control; *NC,* negative control with scrambled siRNA. **, $P < 0.01$.

Bcl-2, and Bcl-xL, in VC312 cells. Sorafenib inhibited expression of cyclins D1/D2/D3 and E and Mcl-1 in VC312 cells (Fig. 4D) in a similar manner to D283 and Daoy cells (Fig. 2).

Sorafenib Rapidly Inhibits STAT3 Phosphorylation at Tyr^{705}

To determine whether inhibition of STAT3 phosphorylation at Tyr^{705} is an early event, we treated Daoy and VC312 cells with 10 μmol/L sorafenib for 5, 15, and 30 min. Western blot analysis (Fig. 5A) showed that the inhibition of phospho-STAT3 Tyr^{705} by sorafenib was detected as early as 5 min after treatment. We also investigated whether sorafenib inhibited phosphorylation of STAT3 at Ser^{727} in tumor cells from medulloblastomas. Our results (Fig. 5B) show that sorafenib did not inhibit the phosphorylation of STAT3 at Ser^{727} in Daoy and VC312 cells.

Sorafenib Inhibits Growth of Human Medulloblastoma Tumor Xenografts in Mice

To examine the effect of sorafenib on growth of medulloblastoma cells *in vivo*, nude mice were inoculated with Daoy cells s.c. and were administered sorafenib by intratumoral injection (100 μL of 10 μmol/L, three times a week). At this dose, no lethal toxicity or weight loss (>10% body weight) was observed among treated animals. Sorafenib significantly inhibited the growth of medulloblastomas in mice as shown in Fig. 6A (52% growth inhibition; $P < 0.001$; $n = 8$). Although sorafenib was only administered three times a week, the inhibitory effect of sorafenib on growth of tumor xenografts was obvious compared with control after 1 week treatment (day 18; Fig. 6A). The expression of total and phosphorylated STAT3 was analyzed in the tumors treated with sorafenib and control tumors. Phosphorylation of STAT3 at Tyr^{705} in two treated tumors was decreased compared with two controls (Fig. 6B), whereas the total STAT3 protein in treated tumors was not decreased. These results provide proof-of-concept that local delivery of sorafenib inhibits the growth of human medulloblastoma tumors associated with decreased tyrosine phosphorylation of STAT3.

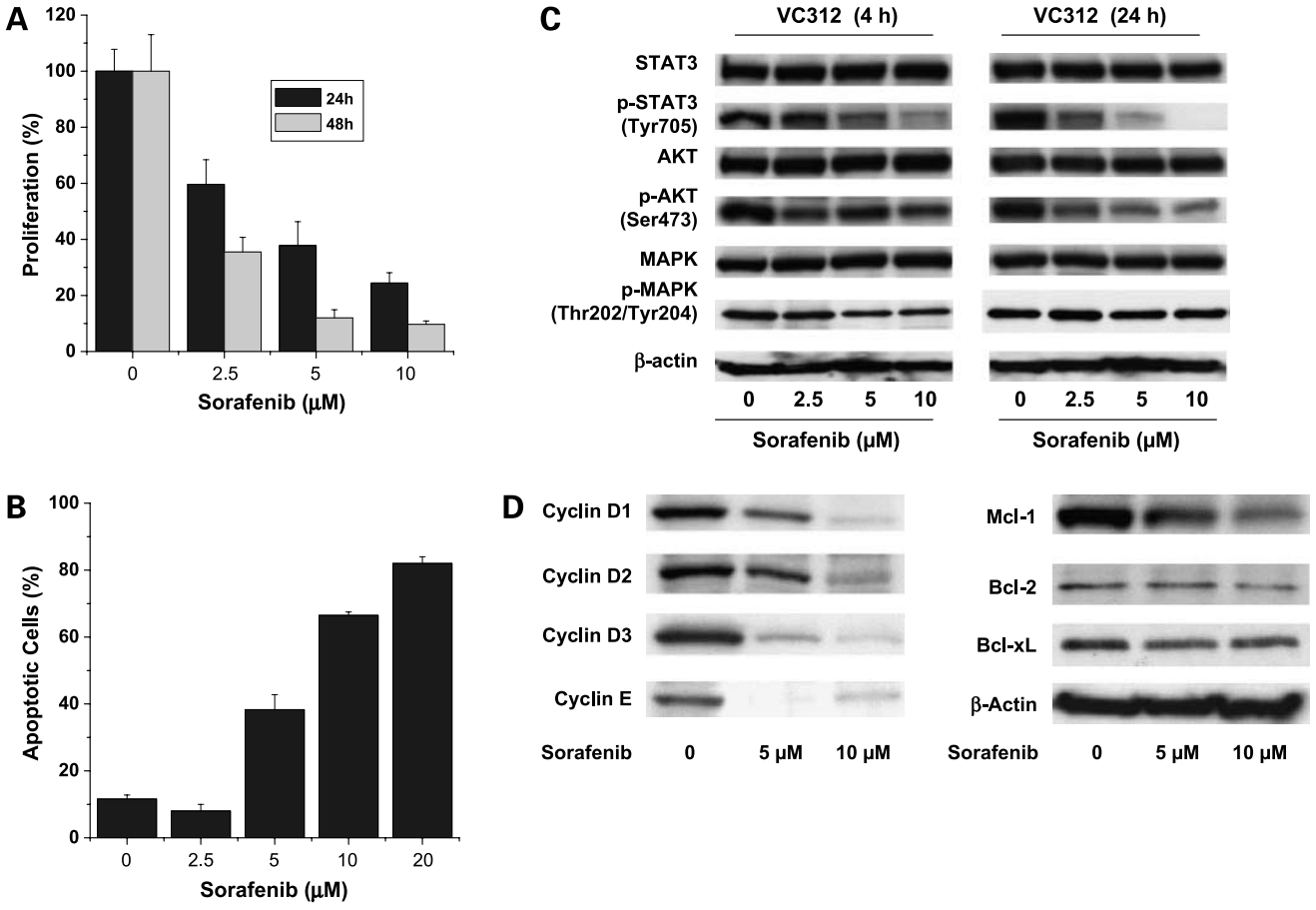

Figure 4. Effects of sorafenib on growth of VC312 cells, signaling pathways, and proteins that control cell cycle and apoptosis. VC312 cells, a primary medulloblastoma culture, were treated with 0, 2.5, 5, or 10 μmol/L sorafenib for 24 and 48 h. **A,** cell proliferation was determined by MTS assay. Apoptosis assays were done after 48 h treatment with sorafenib. Apoptotic cells represented propidium iodide and Annexin V-FITC double-positive cells as determined by flow cytometry (**B**). Western blot analysis in **C** was done with antibodies to total or phosphorylated STAT3, AKT, and MAPK (p44/42). Protein samples from VC312 cells treated with 5 or 10 μmol/L sorafenib for 24 h were also used to determine the expression of cyclins D1, D2, D3, and E and Mcl-1 with specific antibodies (**D**). β-Actin was used as loading control.

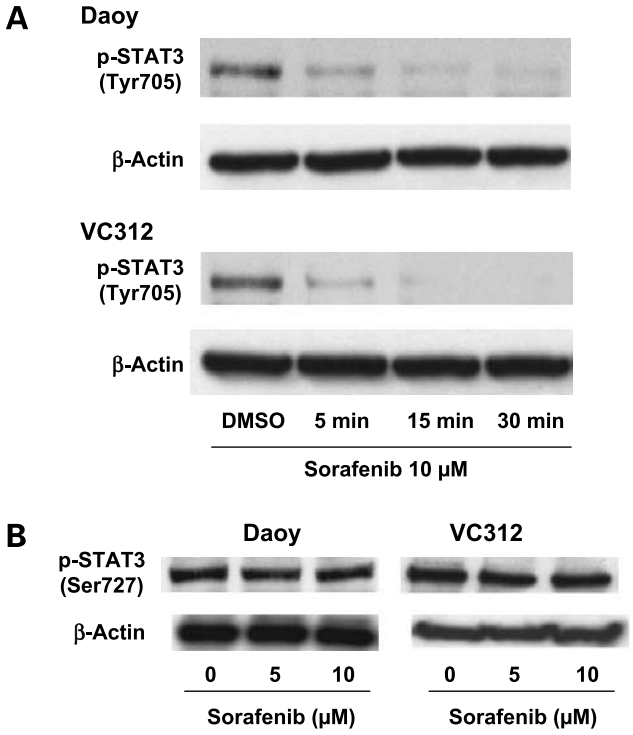

Figure 5. Sorafenib inhibits phosphorylation of STAT3 at Tyr^{705} as early as 5 min after treatment. Daoy and VC312 cells were treated with 10 μmol/L sorafenib for 5, 15, 30 min, respectively. Total cell lysates were prepared and immunoblots were done with antibody to phosphorylated STAT3 at Tyr^{705} (**A**). Phosphorylation of STAT3 at Ser^{727} in Daoy and VC312 cells treated with sorafenib was also examined by immunoblots (**B**). β-Actin serves as loading control.

Discussion

Here, we provide evidence that sorafenib inhibits cell proliferation and survival of two established cell lines (D283 and Daoy) as well as a primary culture (VC312) from human medulloblastomas. Our results suggest that sorafenib inhibits growth of these medulloblastoma cells at least in part through blockade of the STAT3 signaling pathway. Although these tumor cells exhibit different growth properties, inhibition STAT3 phosphorylation at Tyr^{705} in response to sorafenib is common among all of them. Furthermore, inhibition of STAT3 phosphorylation at Tyr^{705} by sorafenib is detectable 5 min after treatment, indicating that the effect of sorafenib on STAT3 phosphorylation is an early and relatively direct event.

STAT3 activation requires Tyr^{705} phosphorylation, resulting in dimerization, nuclear translocation, DNA binding, and transcriptional activation of target genes (23). Phosphorylation of STAT3 at Ser^{727} further enhances transcriptional activation of genes (23). Our data show that total STAT3 proteins as well as phosphorylation on both Tyr^{705} and Ser^{727} were constitutively expressed in biopsies from human medulloblastomas. STAT3 regulates basic biologic processes important in tumorigenesis including cell cycle progression, apoptosis, tumor angiogenesis, and tumor-cell evasion of the immune system (8, 25). Key genes in cell cycle control, such as cyclin D1, are regulated by STAT3 (8). The expression of cyclin D1/D2/D3 and E are decreased by sorafenib in medulloblastoma cells, consistent with the observed inhibition of cell cycle progression.

Expression of Mcl-1, an antiapoptotic protein, is also regulated by STAT3 signaling (8). Mcl-1 has been shown to have a critical role in the survival of malignant cells, especially in leukemia and myeloma (26). Sorafenib has been reported to down-regulate the expression of Mcl-1 in diverse types of tumor cells (27, 28). Here, we show that sorafenib inhibited Mcl-1 expression in both established cell lines and a primary culture of medulloblastomas. Blocking STAT3 protein in human tumor cells has been shown to down-regulate Mcl-1 expression and induce tumor cell apoptosis (8). STAT3 and Mcl-1 are the only proteins inhibited in common among the D283, Daoy, and VC312 cells. Therefore, down-regulation of Mcl-1 by inhibition of phosphorylated STAT3 may be an important mechanism of action of sorafenib in medulloblastomas.

In addition to STAT3 signaling, both Raf-MEK-MAPK and phosphatidylinositol 3-kinase/AKT have important roles in the proliferation and survival of tumor cells (7, 14).

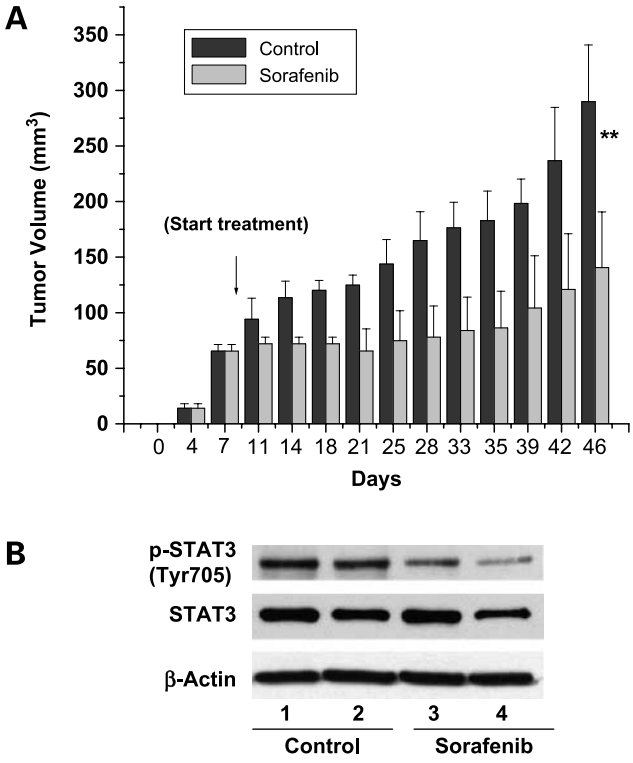

Figure 6. Sorafenib suppresses growth of a human medulloblastoma tumor xenograft in nude mice. Daoy tumor cells (5×10^6 per animal) were implanted s.c. in nude mice as described in Materials and Methods. Treatment was initiated on day 10. Sorafenib (100 μL of 10 μmol/L) was administered by intratumoral injection three times a week. **A,** mean ± SD tumor volume ($n = 8$). Expression of total and phosphorylated STAT3 in two tumors from the treated group and two tumors from the control group were analyzed by immunoblots (**B**). **, $P < 0.001$.

Our data suggest that active AKT might also be suppressed by sorafenib, although MAPK signaling does not appear to be affected under the conditions examined in this study. Because sorafenib inhibits vascular endothelial growth factor receptors (13), key regulators of tumor neoangiogenesis in medulloblastoma (29), it is possible that sorafenib could inhibit angiogenesis in medulloblastoma.

Sorafenib shows good tolerability and promising antitumor activity from clinical trials in several types of solid tumors (20–22). An insidious feature of medullobastomas is their ability to metastasize and disseminate through the cerebrospinal fluid (30). Treatment of medulloblastoma is complicated by the blood-brain barrier, which acts as a physiologic barrier for delivery of drugs to the central nervous system. Various approaches have been developed for local delivery of drugs to brain tumors, including convection-enhanced delivery (31). Thus, local delivery of sorafenib to the cerebrospinal fluid by convection-enhanced delivery may result in more effective antitumor activity with reduced systemic toxicity. In summary, sorafenib is potentially a promising drug for the treatment of pediatric medulloblastomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. McNeil DE, Cote TR, Clegg L, Rorke LB. Incidence and trends in pediatric malignancies medulloblastoma/primitive neuroectodermal tumor: a SEER update. Surveillance Epidemiology and End Results. Med Pediatr Oncol 2002;39:190 – 4.

2. Berman DM, Karhadkar SS, Hallahan AR, et al. Medulloblastoma growth inhibition by hedgehog pathway blockade. Science 2002;297: 1559 – 61.

3. Taylor MD, Liu L, Raffel C, et al. Mutations in SUFU predispose to medulloblastoma. Nat Genet 2002;31:306 – 10.

4. Hallahan AR, Pritchard JI, Hansen S, et al. The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. Cancer Res 2004;64:7794 – 800.

5. Zurawel RH, Chiappa SA, Allen C, Raffel C. Sporadic medulloblastomas contain oncogenic β-catenin mutations. Cancer Res 1998;58:896 – 9.

6. Eberhart CG, Tihan T, Burger PC. Nuclear localization and mutation of β-catenin in medulloblastomas. J Neuropathol Exp Neurol 2000;59: 333 – 7.

7. Wlodarski P, Grajkowska W, Lojek M, Rainko K, Jozwiak J. Activation of Akt and Erk pathways in medulloblastoma. Folia Neuropathol 2006;44: 214 – 20.

8. Yu H, Jove R. The STATs of cancer—new molecular targets come of age. Nat Rev Cancer 2004;4:97 – 105.

9. Darnell JE, Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994;264:1415 – 21.

10. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000;19:2474 – 88.

11. Schaefer LK, Ren Z, Fuller GN, Schaefer TS. Constitutive activation of Stat3α in brain tumors: localization to tumor endothelial cells and activation by the endothelial tyrosine kinase receptor (VEGFR-2). Oncogene 2002;21:2058 – 65.

12. Cattaneo E, Magrassi L, De-Fraja C, et al. Variations in the levels of the JAK/STAT and ShcA proteins in human brain tumors. Anticancer Res 1998;18:2381 – 7.

13. Wilhelm SM, Carter C, Tang L, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 2004;64:7099 – 109.

14. Downward J. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 2003;3:11 – 22.

15. Hilger RA, Scheulen ME, Strumberg D. The Ras-Raf-MEK-ERK pathway in the treatment of cancer. Onkologie 2002;25:511 – 8.

16. Adjei AA. Blocking oncogenic Ras signaling for cancer therapy. J Natl Cancer Inst 2001;93:1062 – 74.

17. Liu L, Cao Y, Chen C, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 2006;66: 11851 – 8.

18. Chang, YS, Adnane J, Trail PA, et al. Sorafenib (BAY 43-9006) inhibits tumor growth and vascularization and induces tumor apoptosis and hypoxia in RCC xenograft models. Cancer Chemother Pharmacol 2007;59:561 – 74.

19. Yu C, Friday BB, Lai JP, et al. Cytotoxic synergy between the multikinase inhibitor sorafenib and the proteasome inhibitor bortezomib *in vitro*: induction of apoptosis through Akt and c-Jun NH_2-terminal kinase pathways. Mol Cancer Ther 2006;5:2378 – 87.

20. Abou-Alfa GK, Schwartz L, Ricci S, et al. Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. J Clin Oncol 2006;24: 4293 – 300.

21. Strumberg D, Clark JW, Awada A, et al. Safety, pharmacokinetics, and preliminary antitumor activity of sorafenib: a review of four phase I trials in patients with advanced refractory solid tumors. Oncologist 2007; 12:426 – 37.

22. Gridelli, C, Maione P, Del Gaizo F, et al. Sorafenib and sunitinib in the treatment of advanced non-small cell lung cancer. Oncologist 2007;12: 191 – 200.

23. Clevenger CV. Roles and regulation of stat family transcription factors in human breast cancer. Am J Pathol 2004;165:1449 – 60.

24. Maddika S, Ande SR, Panigrahi S, et al. Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy. Drug Resist Updat 2007;10:13 – 29.

25. Haura EB, Turkson J, Jove R. Mechanisms of disease: insights into the emerging role of signal transducers and activators of transcription in cancer. Nat Clin Pract Oncol 2005;2:315 – 24.

26. Mandelin AM II, Pope RM. Myeloid cell leukemia-1 as a therapeutic target. Expert Opin Ther Targets 2007;11:363 – 73.

27. Rahmani M, Davis EM, Bauer C, Dent P, Grant S. Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. J Biol Chem 2005;280:35217 – 27.

28. Yu C, Bruzek LM, Meng XW, et al. The role of Mcl-1 downregulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. Oncogene 2005;24:6861 – 9.

29. Slongo ML, Molena B, Brunati AM, et al. Functional VEGF and VEGF receptors are expressed in human medulloblastomas. Neuro-oncol 2007; 9:384 – 92.

30. Fouladi M, Gajjar A, Boyett JM, et al. Comparison of CSF cytology and spinal magnetic resonance imaging in the detection of leptomeningeal disease in pediatric medulloblastoma or primitive neuroectodermal tumor. J Clin Oncol 1999;17:3234 – 7.

31. Sawyer AJ, Piepmeier JM, Saltzman WM. New methods for direct delivery of chemotherapy for treating brain tumors. Yale J Biol Med 2006; 79:141 – 52.