INTRODUCTION

Neuroblastoma is a clinically heterogeneous pediatric malignancy. Approximately half of the 650 new cases of neuroblastoma diagnosed each year in the United States meet the Children’s Oncology Group criteria for high-risk disease [1]. Although the majority of high-risk tumors are initially chemotherapy responsive, relapse rates, and therapy-related toxicities are high, rendering long-term survival dismal [2]. Thus, novel therapies are desperately needed for this group of patients.

Angiogenesis has been shown to play a critical role in the growth and metastasis of all malignant tumors [3], and increased vascular density and microvascular proliferation are associated with clinically aggressive disease and poor prognosis in neuroblastoma [4–6]. Anti-angiogenic agents have been validated as a therapeutic option in adult malignancies [7], and anti-tumor effects have been observed in preclinical neuroblastoma models [8–10]. We hypothesized that the multikinase inhibitor, sorafenib, would target both neoplastic cells and endothelial cells, leading to increased therapeutic benefit when compared to agents that target angiogenesis alone. Sorafenib inhibits autophosphorylation of growth factor receptors, c-Kit, and RET receptors, which are critical for pro-angiogenic signaling [11]. Recently, sorafenib has been shown to inhibit HIF-1α upregulation in neuroblastoma cell lines [12], which may further amplify its anti-angiogenic activity in neuroblastoma tumors. Sorafenib also competitively inhibits B-Raf and C-Raf activation, resulting in attenuation of the pro-angiogenic mitogen activated protein kinase (MAPK) signaling pathway [13].

In this study we analyzed the effects of sorafenib on neuroblastoma angiogenesis and tumor growth in subcutaneous xenograft and orthotopic adrenal neuroblastoma preclinical models. We also investigated the direct effects of sorafenib on neuroblastoma cell signaling and proliferation. Our studies show that sorafenib potently inhibits angiogenesis and neuroblastoma tumor growth in both preclinical models. We also confirm that sorafenib mediates direct changes in neuroblastoma cell signaling, resulting in inhibited cell cycle and proliferation.

METHODS

Cell Lines

MYCN-amplified, human neuroblastoma cell lines NBL-W-N, SMS-KCNR, and LA1-55n were grown at 5% CO₂ in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin. NBL-W-N was established in our laboratory [14], SMS-KCNR was a kind gift from Dr. Carol Thiele, and LA1-55n a kind gift from Dr. June Biedler. Cell lines were authenticated by short tandem repeat (STR) profiling using the AmpFlSTR Identifier PCR Amplification Kit (Applied Biosystems, Carlsbad, CA). Analysis was performed at The Johns Hopkins University Fragment Analysis Laboratory.

Background. More effective therapy for children with high-risk neuroblastoma is desperately needed. Preclinical studies have shown that neuroblastoma tumor growth can be inhibited by agents that block angiogenesis. We hypothesized that drugs which target both neuroblastoma cells and tumor angiogenesis would have potent anti-tumor activity. In this study we tested the effects of sorafenib, a multi-kinase inhibitor, on neuroblastoma cell proliferation and signaling, and in mice with subcutaneous human neuroblastoma xenografts or orthotopic adrenal tumors. Procedure. Mice with subcutaneous neuroblastoma xenografts or orthotopic adrenal tumors were treated with sorafenib, and tumor growth rates were measured. Blood vessel architecture and vascular density were evaluated histologically in treated and control neuroblastoma tumors. The in vitro effects of sorafenib on neuroblastoma proliferation, cell cycle, and signaling were also evaluated.

Results. Sorafenib inhibited tumor growth in mice with subcutaneous and orthotopic adrenal tumors. Decreased numbers of cycling neuroblastoma cells and tumor blood vessels were seen in treated versus control tumors, and the blood vessels in the treated tumors had more normal architecture. Sorafenib treatment also decreased neuroblastoma cell proliferation, attenuated ERK signaling, and enhanced G1/G0 cell cycle arrest in vitro. Conclusions. Our results demonstrate that sorafenib inhibits the growth of neuroblastoma tumors by targeting both neuroblastoma cells and tumor blood vessels. Single-agent sorafenib should be evaluated in future phase II neuroblastoma studies.
Facility (Baltimore, MD) and STR profiles were found to be identical to known profiles for the cell lines.

In Vivo Neuroblastoma Models

To establish neuroblastoma subcutaneous xenografts, female nude mice (Harlan, Madison, WI) underwent subcutaneous injection of $1 \times 10^7$ SMTS-KCNR neuroblastoma cells as previously described [15]. Once tumors reached a palpable size of approximately 70 mm$^3$, mice were treated by oral gavage with vehicle control (5% Cremophor EL/5% ethanol/90% ddH$_2$O) or 45 mg/kg sorafenib tosylate (30 mg/kg free base equivalent), a dosage found to be effective and well tolerated in multiple preclinical models of cancer [13]. Sorafenib was prepared as previously described [16], and formulated fresh daily at 4X the highest dose in a Cremophor EL/ethanol (50:50) solution. Final dosing solutions were prepared on the day of use by dilution to 1X with ddH$_2$O and mixed immediately before dosing. Mice were treated 1X/day, 5 days/week for a total of 8 doses over 10 days and sacrificed when control tumors reached terminal size. Tumor size was determined every 2–3 days by external measurements with a caliper and volumes were calculated using the formula: $\text{Tumor volume} = (\text{length} \times \text{width}^2)/2$.

To generate orthotopic tumors, SMTS-KCNR cells were regrafted into the adrenal gland of nude mice. A left side parasternal approach was taken and the spleen was displaced cranially allowing exposure of the left adrenal gland. 2 $\times 10^6$ SMTS-KCNR cells in 20 $\mu$L of RPMI with 10% FBS were injected through a 27 G needle into the adrenal gland. Flank musculature was closed with a single 4–0 absorbable suture and the skin closed with 3 or 4 staples. Due to the complexity of the procedure, injections were performed over 2 days. Animals were randomized 7 days after injection and treatment was started at that time. The animals were all sacrificed on the same day, after 16 or 17 days. In the test group, 7 animals received 16 doses; 6 received 17 doses. In the control group, 2 received 16 doses; 5 received 17. Animals were weighed 3X/week and sacrificed when mice in the control group, 2 received 16 doses; 5 received 17 doses. In the control group, 7 animals received 16 doses; 6 received 17 doses. In the test group, 7 animals received 16 doses; 6 received 17 doses. In the control group, 2 received 16 doses; 5 received 17. Animals were weighed 3X/week and sacrificed when mice in the control group showed signs of distress due to tumor burden, such as stooped posture, inability to stand, and loss of skin turgor. Tumors were removed, measured, weighed, and photographed. Tissue was fixed with 10% buffered formalin, embedded in paraffin and 5 $\mu$m-thick sections were prepared for histologic evaluation. Animals were treated according to NIH guidelines for animal care and use, and protocols approved by the University of Chicago Institutional Animal Care and Use Committee.

Histological Analysis and Immunofluorescence

Neuroblastoma xenografts and orthotopic tumors were processed for histologic evaluation, stained with hematoxylin and eosin (H&E), and endothelial cells were detected with anti-human CD31 antibody as described [6]. For immunofluorescence analysis, endothelial cells were stained with anti-CD31 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:50 dilution and FITC-labeled secondary antibody. Pericytes were visualized with anti-$\alpha$-SMA (Sigma, St. Louis, MO) at 1:100 dilution and Texas Red-labeled secondary antibody as described [17]. For analysis of proliferation rate, slides were incubated with Ki-67 mouse anti-human monoclonal antibody (clone MIB-1, 1:200; DakoCytomation, Carpinteria, CA) as previously described [6].

Quantification of Vascular Density and Architecture

Tumor sections stained for CD31 were examined at x400 magnification. The number of CD31 positive cells per high power field were counted in triplicate by two blinded investigators and then averaged. Two-dimensional shape descriptors of Image J software were used to quantitatively assess blood vessel architecture, and the abnormality of blood vessel structure was graded using the formula $1 - (\text{circularity} \times \text{roundness} \times \text{solidity})$ as described [18].

Proliferation Assay

The CellTiter 96 AQueous Non-radioactive Proliferation Assay kit (Promega, Madison, WI) was used as previously described [15]. Briefly, cells were seeded and after 24 hours sorafenib was added to triplicate wells at concentrations from 0 to 100 $\mu$M. Following a 96 hours incubation, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was added and absorbance measured using a Synergy 2 microplate reader (Bio-Tek Instruments, Winooski, VT).

Measurement of Cell Cycle Phase Distribution

Neuroblastoma cell lines were cultured with 10 $\mu$M sorafenib or vehicle control for 24 hours. Following treatment, cells were washed with phosphate buffered saline (PBS), fixed in 70% ethanol, and stored at 4°C. Cells were hypotonically lysed in 1 ml of 0.05 mg/ml propidium iodide (Sigma) and 0.1% Triton X-100, and data analyzed on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA) using Flowjo software (Tree Star, Ashland, OR).

Western Blot Analysis for Extracellular Signal-Regulated Kinase (ERK), Phospho-ERK (pERK), and Cyclins D1, D2, D3, and E

NBL-W-N, SMS-KCNR, and LA1-55n neuroblastoma cells were treated with 5 and 10 $\mu$M sorafenib for 24 hours. Lysates were prepared by boiling cell pellets in buffer containing 50 mM TRIS-HCl (pH 6.8), 2% SDS, and protease inhibitor cocktail (Sigma) for 10 min. Total protein (10 $\mu$g) was electrophoresed on 4–20% SDS-PAGE gradient gels and transferred to nitrocellulose membranes. Antibodies against cyclins D1, D2, D3, and E were obtained from BD Pharmingen, San Diego, CA, and antibodies against p44/42 MAPK (Erk1/2), and phospho-p44/42 MAPK (pErk1/2, Thr202/Tyr204) were obtained from Cell Signaling Technologies, Beverly, MA. Membranes were blocked in Tris-buffered saline (TBS) with 0.1% Tween-20 and 5% non-fat dry milk (ERK and cyclins) or TBS with 0.1% Tween-20 and 5% bovine serum albumin (BSA) for pERK. Blots were developed with anti-mouse or anti-rabbit horseradish peroxidase secondary antibodies (KPL, Gaithersburg, MD) and Immun-Star Western C Detection Kit (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

All in vitro experiments were repeated at least in triplicate and standard deviations were calculated. At least five mice per group were included in each animal experiment and mean values of the tumor volumes, weights, and vessel densities were compared. All
the quantitative values obtained in the experiments were evaluated using paired Student's t-test. A P-value of 0.05 was considered statistically significant. A nonlinear regression, sigmoidal four-parameter dose-response model was used to determine IC50 by plotting log(inhibitor concentration) versus relative survival after 96-hours treatment with sorafenib. Prism software (GraphPad Software, Inc., La Jolla, CA) was utilized to determine the IC50.

RESULTS

Sorafenib Inhibits Neuroblastoma Tumor Growth In Vivo

After 4 days of sorafenib treatment, the average size of subcutaneous xenografts in the animals receiving sorafenib was significantly smaller compared to tumors in control mice (376.3 ± 199.5 mm³ vs. 685.9 ± 207.2 mm³, respectively; \( P = 0.033 \)). The mice were sacrificed after receiving eight doses of sorafenib over 10 days, when control tumors reached terminal size. As shown in Figure 1A, after 10 days of treatment, the average size of the tumors in the sorafenib-treated mice was approximately 25% of the size of the tumors in controls (541.0 ± 320.6 mm³ vs. 2317.1 ± 773.1 mm³, respectively, \( P < 0.001 \)).

Because orthotopic tumors have been shown to be superior models of tumor angiogenesis [19], we also tested the effects of sorafenib in mice with adrenal orthotopic neuroblastomas. Treatment was initiated 7 days after tumor cell inoculation, and continued for 16–17 doses. The animals were sacrificed when signs of tumor burden distress appeared in the control group. We again found that tumors in the animals treated with sorafenib were significantly smaller than tumors in the controls (538.6 ± 376.3 mm³ vs. 1615.6 ± 753.4 mm³, respectively; \( P < 0.001 \), Fig. 1B). Similarly, the average tumor weight in treated versus control animals was significantly less (0.5 ± 0.2 g vs. 1.6 ± 0.7 g, respectively; \( P < 0.0001 \), Fig. 1C).

Sorafenib Inhibits Neuroblastoma Tumor Angiogenesis

To investigate the effects of sorafenib treatment on angiogenesis, we counted the number of endothelial cells in xenograft tumor sections immunostained with anti-CD31 antibody. As shown in Figure 2A and B, the average number of CD31 positive cells per high power field was significantly lower in the sorafenib-treated tumors compared to controls (79.4 ± 9.9 vs. 106.8 ± 10.1, respectively; \( P = 0.0008 \)). We used immunofluorescent staining and Image J software to further characterize blood vessel number and architecture, and to assign quantitative values relevant to blood vessel architecture as previously described [18]. Circularity is a function of shape, roundness measures elongation, and solidity approximates the density. Blood vessel number and architecture, and to assign quantitative values obtained in the experiments were evaluated using paired Student’s t-test. A P-value of 0.05 was considered statistically significant. A nonlinear regression, sigmoidal four-parameter dose-response model was used to determine IC50 by plotting log(inhibitor concentration) versus relative survival after 96-hours treatment with sorafenib. Prism software (GraphPad Software, Inc., La Jolla, CA) was utilized to determine the IC50.

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of SMS-KCNR cells revealed an increase in G1 arrested cells from 50% to 70% with 10 μM sorafenib treatment for 24 hours (P = 0.03). Sorafenib also induced an increase in G1 arrested NBL-W-N (60–80%; P = 0.04). However, no significant increase in the percentage of G1 arrested cells was seen in experiments with LA1-55n cells (Fig. 4B).

**Sorafenib Attenuates ERK Signaling and Cyclin D1 Expression in Human Neuroblastoma Cell Lines**

To examine the effects of sorafenib on MAPK pathway signaling in neuroblastoma cells, we performed western blot analyses using anti-ERK and anti-pERK antibodies. ERK phosphorylation was inhibited by 5 and 10 μM sorafenib after 24 hours of treatment in all three neuroblastoma cell lines (Fig. 5). We also found that sorafenib treatment profoundly decreased expression of the downstream protein cyclin D1 in all three human neuroblastoma cell lines. Levels of cyclins D2 and E were also decreased, however, total ERK expression was unchanged (Fig. 5).

**DISCUSSION**

In this study, we investigated the anti-tumor activity of the multi-kinase inhibitor sorafenib, utilizing two preclinical...
Sorafenib induces G1 arrest in neuroblastoma cell lines. A: Treatment with increasing concentrations of sorafenib reduced the survival of three MYCN-amplified human neuroblastoma cell lines, as measured by the MTS assay. The IC50 of each cell line is indicated. B: Propidium iodide staining and flow cytometry were utilized to determine cell cycle distribution. Sorafenib increased the percentage of G1-arrested SMS-KCNR (P < 0.001) and NBL-W-N (P < 0.001) cells. No significant increase in the number of G1-arrested LA1-55n cells was seen with sorafenib treatment (P = 0.329). Bars represent percentage of cells in indicated cell cycle phase after 24 hours of treatment with 10 μM sorafenib. Statistically significant differences from the control group are marked with an asterisk.

Sorafenib is a multikinase inhibitor which was recently approved by the Food and Drug Administration (FDA) for treatment of renal cell carcinoma and hepatocellular carcinoma [13], and a Phase I pediatric trial has recently been completed by the Children’s Oncology Group (COG) in patients with refractory solid tumors and leukemia [22]. In the pediatric study, no grade 3 hypertension was seen at the maximal tolerated dose of 200 mg/m², and only 1/6 patients experienced a grade 3 dose limiting toxicity of elevated liver enzymes. However, clinical data on the effect of sorafenib in neuroblastoma are not yet available as no reference to neuroblastoma patients was included in this trial.

Others have also shown that sorafenib has anti-neuroblastoma activity in preclinical studies. Roy et al. [23] showed that the growth of two neuroblastoma cell lines is inhibited in vitro after treatment with sorafenib. Further, Chai et al. [24] demonstrated that sorafenib decreases ERK, Akt1/2/3, AMPK, and STAT3 phosphorylation in a single neuroblastoma cell line. More recently, Keir et al. [20] reported results from the Pediatric Preclinical Testing Program (PPTP) demonstrating that sorafenib inhibited the proliferation of a number of pediatric cancer cell lines including three of four human neuroblastoma cell lines. Although no mice were cured by sorafenib, decreased tumor growth and prolonged event-free survival were seen with sorafenib treatment [20]. Interestingly, the IC50 for the more resistant LA1-55n cells used in our study was equal to the median of the entire panel analyzed by the PPTP. Thus, SMS-KCNR and NBL-W-N appear to be more sensitive than the other cell lines tested in this panel. It is well established that significant differences in tumor blood vessel density are seen in subcutaneous versus orthotopic preclinical tumor models [19]. The orthotopic adrenal neuroblastoma model used in our studies more closely mimics the blood supply of clinical tumors, and the activity of sorafenib in this model further emphasizes its potential clinical utility.

By competitively inhibiting B-Raf and C-Raf activation, sorafenib inhibits the pro-oncogenic MAPK signaling pathway [25]. Insulin growth factor 1 (IGF-1) activates this pathway in neuroblastoma, and Misawa et al. [26] showed that following IGF-1 treatment of KP-N-RT human neuroblastoma cells, cell cycle progression was stimulated and MYCN expression was up-regulated. Further, experiments demonstrated that MAPK pathway activation was required for the induction of MYCN expression and cell proliferation, as treatment with a MEK1 inhibitor blocked these effects. Sandoval et al. [27] similarly demonstrated the anti-
tumor re-growth when given in the setting of minimal residual disease [8,9]. The promising results seen in the preclinical studies conducted by our group and others, together with the low toxicity profile observed in early phase clinical trials, indicate that sorafenib may have therapeutic utility in children with neuroblastoma. Further preclinical studies testing the efficacy of combination therapy and the optimal sequence of administration of the combination therapies are warranted.

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