Thrombospondin-1 Peptide ABT-510 Combined with Valproic Acid Is an Effective Antiangiogenesis Strategy in Neuroblastoma

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Abstract

In the pediatric cancer neuroblastoma, clinically aggressive disease is associated with increased levels of angiogenesis stimulators and high vascular index. We and others have hypothesized that blocking angiogenesis may be effective treatment for this pediatric malignancy. However, little is known about the efficacy of antiangiogenic agents in pediatric malignancies. Recently, promising results have been reported in an adult phase I study of ABT-510, a peptide derivative of the natural angiogenic inhibitor thrombospondin-1. Histone deacetylase inhibitors, such as valproic acid (VPA), have also been shown to have antiangiogenic activity in several cancer models. In this study, we evaluated the effects of ABT-510 and VPA on neuroblastoma tumor growth and angiogenesis. Although only VPA was capable of blocking the proliferation of neuroblastoma cells and inducing neuroblastoma cell apoptosis in vitro, treatment with VPA or ABT-510 alone significantly suppressed the growth of neuroblastoma xenografts established from two different MYCN-amplified cell lines. Combination therapy more effectively inhibited the growth of small neuroblastoma xenografts than single-agent treatment, and in animals with large xenografts, total cessation of tumor growth was achieved with this treatment approach. The microvascular density was significantly reduced in the xenografts treated with combination therapy compared with controls or tumors treated with single agents. In addition, the number of structurally abnormal vessels was reduced, suggesting that these agents may “normalize” the tumor vasculature. Our results indicate that ABT-510 combined with VPA may be an effective antiangiogenic treatment strategy for children with high-risk neuroblastoma. [Cancer Res 2007;67(4):1716–24]

Introduction

The clinical hallmark of neuroblastoma is heterogeneity, with the likelihood of tumor progression varying widely according to age at diagnosis and extent of disease (1). In addition to these clinical factors, biological markers, such as MYCN gene status, tumor cell ploidy, and tumor histology, have also been shown to be strongly predictive of risk of relapse (2). Modern treatment strategies are stratified according to these clinical and biological classifiers, and substantial progress has been made in the treatment of children with low- and intermediate-risk neuroblastoma with reduced therapy approaches. However, more effective therapy is still needed for children with high-risk disease. Despite intensive multimodality treatment, <30% of high-risk patients are cured (1). Similar to other solid tumors, angiogenesis is required for neuroblastoma tumor growth and metastasis. The majority of published studies indicate that angiogenesis contributes to the aggressive behavior of neuroblastoma tumors, and a positive correlation between poor outcome and high levels of factors that stimulate blood vessel growth and vascular index has been reported (3, 4). In addition, preclinical experiments have shown that angiogenesis inhibitors are capable of suppressing neuroblastoma growth, further supporting the concept that the blood vessels in neuroblastoma tumors are likely to be clinically relevant therapeutic targets (5, 6).

Thrombospondin-1 (TSP-1) is a well-known natural inhibitor of angiogenesis, and down-regulation of TSP-1 plays a critical role in the angiogenic switch in several tumor types (7–9). In neuroblastoma, we have previously shown that TSP-1 is epigenetically silenced in a subset of undifferentiated, advanced-stage tumors and cell lines, whereas it is expressed in tumors with morphologic evidence of neuroblast differentiation (10). The antiangiogenic effect of TSP-1 is mediated in part by the CD36 receptor, which triggers a signaling cascade that leads to apoptosis in activated endothelial cells via the CD95 death receptor. The inhibitory activity of TSP-1 has been mapped to the properdin type 1 repeats in the NH2-terminal third of the molecule, and a single substitution of d-isoleucine for l-isoleucine in one properdin-region heptapeptide leads to a 1,000-fold increase in the antiangiogenic activity (11, 12). ABT-510 is a modified analogue of the active TSP-1 heptapeptide, which has slowed tumor growth in both xenograft and syngeneic models (13). Administration of ABT-510 to TSP-1 knockout mice for 5 consecutive days has been shown to reduce by ~5-fold both elevated circulating endothelial cells and endothelial precursor cells to near wild-type levels (14). Although no pediatric cancer studies have been done to date, a recently completed phase I trial of ABT-510 in adults with advanced cancer has shown a favorable toxicity profile and a decrease in the median serum basic fibroblast growth factor (bFGF) levels. Stable disease lasting for six cycles or more was seen in 6 of the 39 patients enrolled on the phase I study, and additional response data are being collected on ongoing phase II studies (15).

Valproic acid (VPA), a branched chain fatty acid initially used for the treatment of epilepsy, has also been shown to have anticancer activity and is currently being tested in clinical trials. VPA inhibits the growth of many types of cancer, including glioma, neuroblastoma (16, 17), breast cancer (18), acute myelogenous leukemia (19, 20), thyroid cancer (21), and endometrial carcinoma (22). Recently, VPA and IFN-α have been reported to synergistically inhibit neuroblastoma growth, and increased anticancer activity...
has also been seen when VPA is combined with all-trans retinoic acid (16, 17). Although the mechanisms responsible for the anti-cancer activity of VPA are not completely understood, like other short chain fatty acids, it inhibits histone deacetylase (HDAC) activity and plays a role in the regulation of gene expression. Recent studies have indicated that HDAC inhibitors are also capable of blocking hypoxia-induced and vascular endothelial growth factor–induced angiogenesis (23).

In this study, we investigated the effects of the antiangiogenic agent ABT-510 on neuroblastoma growth and angiogenesis. Because the efficacy of angiogenesis inhibitors is greatly improved when they are combined with other anticancer drugs (24, 25), we conducted additional experiments with ABT-510 combined with VPA. VPA was selected for the combination studies because it is capable of inhibiting neuroblastoma cell proliferation and tumor growth and can block HDAC activity and angiogenesis. In addition, VPA is known to have a low toxicity profile. We found that VPA, but not ABT-510, was capable of blocking the proliferation of neuroblastoma cells and inducing neuroblastoma cell apoptosis in vitro. However, treatment with either ABT-510 or VPA alone significantly suppressed the growth of neuroblastoma xenografts established from two different MYCN-amplified cell lines. ABT-510 combined with VPA more effectively inhibited the growth of small tumors than single-agent therapy, and in animals with large tumors, combination therapy stabilized tumor growth. In addition to blocking angiogenesis, tumor cell apoptosis and inhibited tumor cell proliferation were seen in the tumors treated with combination therapy. Our results, together with the known low toxicity profile of both agents, suggest that VPA combined with ABT-510 may be an effective, well-tolerated antiangiogenic treatment strategy for children with high-risk neuroblastoma.

Materials and Methods

Cell lines. The biological and genetic characteristics of the SMS-KCN and NMB MYCN-amplified neuroblastoma cell lines used in this study have been previously described (10). Cells were grown at 5% CO2 in RPMI 1640 (Invitrogen, Carsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), l-glutamine, and antibiotics.

Peptide. ABT-510 (Abbott Laboratories, Abbott Park, IL) is a capped Peptide.

Endothelial cell migration assay. Endothelial cell migration assays were carried out in a modified Boyden chamber (NeuroProbe, Gaithersburg, MD) with human microvascular endothelial cells (Cambrex Corp., East Rutherford, NJ) in EBM medium (Cambrex) containing 0.01% bovine serum albumin as described previously (26, 27). Test substances were assayed with or without 5 ng/mL bFGF (National Cancer Institute Preclinical Repository, Bethesda, MD). To generate dose-response curves, the data were normalized as percentage of maximum migration using the difference between bFGF/EBM-induced migration and background migration in EBM alone as 100% control. The experiment was done in triplicate.

CtDNA synthesis and Taqman assay. Total RNA (2 μg) was used for reverse transcription using SuperScript III (Invitrogen) and 50 μmol/L oligo(dT)20 in a 20 μL reaction volume according to standard protocols supplied by the manufacturer. cDNA synthesis was done at 50 °C for 50 min. Following heat inactivation at 85 °C for 5 min and RNase H treatment at 37 °C for 20 min, 480 μL distilled water was added and 3 μL of diluted cDNA were used for Taqman analysis. Taqman reactions were set up containing 1× Taqman Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 250 nmol/L FAM-labeled specific probe, and 900 nmol/L forward and reverse primers in a 20 μL reaction. All assays were carried out in a 96-well format. Real-time fluorescent detection of PCR products was done with an Applied Biosystems 7500 Fast Real-Time PCR System using the following thermocycling conditions: 1 cycle of 50°C for 2 min and 95°C for 20 s; 40 cycles of 94°C for 20 s and 60°C for 1 s.

In situ L-distilled water was added and 3 μL were used for reverse transcription using SuperScript III (Invitrogen) and 50 μmol/L oligo(dT)20 in a 20 μL reaction volume according to standard protocols supplied by the manufacturer. cDNA synthesis was done at 50 °C for 50 min. Following heat inactivation at 85 °C for 5 min and RNase H treatment at 37 °C for 20 min, 480 μL distilled water was added and 3 μL of diluted cDNA were used for Taqman analysis. Taqman reactions were set up containing 1× Taqman Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 250 nmol/L FAM-labeled specific probe, and 900 nmol/L forward and reverse primers in a 20 μL reaction. All assays were carried out in a 96-well format. Real-time fluorescent detection of PCR products was done with an Applied Biosystems 7500 Fast Real-Time PCR System using the following thermocycling conditions: 1 cycle of 50°C for 2 min and 95°C for 20 s; 40 cycles of 95°C for 3 s and 60°C for 30 s. The sequences for the primers and probes for MYCN, TSP-1, SPARC, and β2-microglobulin were described previously (10, 27, 28). Data were analyzed using the comparative method (ΔΔCt) to calculate relative quantities of a nucleic acid sequence. Nontreated SMS-KCN and NMB were used as the calibrator sample, and β2-microglobulin was used as an endogenous control detector.

Neuroblastoma xenograft studies. Four- to 6-week-old female homozygous athymic nude mice (Harlan, Madison, WI) were inoculated s.c. into the right flank with 1 × 106 SMS-KCN or 1.25 × 107 NMB cells. Once tumors were palpable (70 mm3), mice were treated once daily for 20 days with either ABT-510 (i.p., 40 mg/kg), VPA (i.p., 400 mg/kg), or ABT-510 and VPA in combination. In separate experiments, treatment was started after the animals developed larger tumors >380 mm3 in size and continued for 10 days. Tumor volumes were measured twice weekly and calculated using the following formula: tumor volume (length × width2)/2 (29). The Student’s t test was used to compare tumor size in the control and treatment groups. Mice were sacrificed after 20 or 10 days of treatment, respectively. Animals were treated according to NIH guidelines for animal care and use, and protocols were approved by the Animal Care and Use Committee at Northwestern University.

Quantification of apoptosis. In situ detection of cleaved, apoptotic DNA fragments was done using the In Situ Cell Death Detection Kit (Roche Diagnostics Corp., Indianapolis, IN) as described previously (30). Sections of human colon mucosa processed similarly to the xenografts were included in each assay. To check the intra-assay and interassay consistency, the apoptotic bodies in colonic mucosa, stained as the first and last slice of each assay, were assessed. The assays were considered adequate only when the frequency of apoptosis on the two sections of colonic mucosa was

The experiment was done in duplicate. The cell cycle data were analyzed with an Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL), with System II (version 3.0) software (Beckman Coulter). Further analysis of cell cycle distribution was determined by using ModFit LT (Verity Software House, Topsham, ME).
similar. Each terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) in situ–labeled section was quantified using a Leica (Heidelberg, Germany) DM IRB inverted fluorescence microscope equipped with Image-Pro Plus version 4.5 software (Media Cybernetics, Silver Spring, MD) at a magnification of ×400. The frequency of labeled apoptotic cells was obtained by quantifying 10 consecutive fields starting with areas with the highest number of TUNEL-labeled nuclei, avoiding areas of necrosis, and expressed as apoptosis per 10 high-power fields (HPF).

**Immunohistochemical studies.** Xenograft tissue was fixed in 10% formaldehyde/zinc fixative (Electron Microscopy Sciences, Hatfield, PA) and embedded in paraffin. Serial sections (4 μm thick) were deparaffinized and rehydrated as described previously (31). Sections of each tumor were stained with H&E, and selected sections were stained with Masson's trichrome special staining. Additional sections were used for immunohistochemistry. Antigen retrieval was done with 0.01 mol/L citrate buffer (pH 6.0) for Ki-67 or 1 mmol/L EDTA (pH 8.0) for CD31 antibody, heated in a boiling steamer for 20 min, and then cooled down to room temperature for 20 min. For analysis of proliferation rate, slides were incubated with Ki-67 (clone MIB-1, 1:200; DakoCytomation, Carpinteria, CA) mouse anti-human monoclonal antibody. CD31 (platelet/endothelial cell adhesion molecule 1, M-20, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal goat anti-mouse antibody was used to highlight endothelial cells. Primary antibodies were incubated in a humidity chamber overnight at 4°C. The staining for Ki-67 was developed using the DAKO Envision+ System-HRP (DakoCytomation) according to the manufacturer's instructions. The CD31 goat anti-mouse primary antibody was bridged by biotinylated anti-goat IgG (1:200) and detected with streptavidin (1:400; Vector Laboratories, Burlingame, CA). Sections were counterstained with Gill's hematoxylin. In addition, the primary antibodies were omitted in each assay to ensure the specificity. Immunohistochemistry for TSP-1 expression was done as described previously (10). Mean vascular density (MVD) was quantified by measuring pixels in 10 consecutive fields at ×200 magnification.

**Morphologic evaluation of blood vessels.** Blood vessel morphology and tumor histology were evaluated by a pathologist (R.Z.) on H&E-stained sections. The presence or absence of hemorrhage was evaluated, and tumor blood vessels were classified morphologically into four types. Vessels were counted, and the percentage of each vessel type in the tissue section was calculated. Type 1 vessels had disorganized cellular proliferation with poorly formed lumina and vascular endothelial proliferation (VEP). Type 2 vessels were structurally more normal, thin walled, and capillary like. Type 3 vessels were structurally similar to type 2 vessels but were embedded in fibrocollagenous stroma. Type 4 vessels had concentric layers of cells within a thickened wall often accompanied by fibrinoid necrosis and vasculitis.

**Figure 1.** Analysis of SMS-KCNCR cell apoptosis, necrosis, cell cycle, and endothelial cell migration after treatment with ABT-510 or VPA. A, flow cytometric analysis of SMS-KCNCR cell apoptosis and necrosis after treatment with ABT-510 for 1 d. Top left quadrant, necrotic cells; top right quadrant, late apoptotic cells; bottom right quadrant, early apoptotic cells; bottom left quadrant, live cells. B, sub-G1 apoptotic analysis of SMS-KCNCR cells treated with VPA. C, cell cycle analysis of control, ABT-510–treated, and VPA-treated neuroblastoma cells. SMS-KCNCR was treated with either vehicle or various concentrations of ABT-510 and VPA for 24 h and analyzed by flow cytometry. D, ABT-510 and VPA inhibited angiogenesis in vitro.
Valproic Acid and TSP-1 Inhibit Neuroblastoma Growth

Evan’s blue dye assay. The Evan’s blue dye assay was used to assess tumor microvascular permeability as described previously (32). Evan’s blue dye (2%, 100 μL; MP Biomedicals, Aurora, OH) was given via tail vein injection of tumor-bearing mice and allowed to circulate for 20 min. To remove remaining intravascular dye, mice were perfused with 25 mL saline through the left ventricle with a right atrial vent. Tumors were excised, cut into pieces, and weighed. Formaldehyde was added at a ratio of 1 mL/100 mg of tissue and left stand for 72 h at room temperature to facilitate the extraction of Evan’s blue dye. Tissues were then removed and resultant extract was centrifuged. The levels of Evan’s blue were quantified using a spectrophotometer at a wavelength of 620 nm. The amount of Evan’s blue dye remaining in the tumor reflects the degree of intratumoral vessel permeability. All samples were run in duplicate and compared with those of standards.

Statistical analysis. The two-tailed Student’s t test was used to compare the statistical significance of differences between the mean values of quantification of apoptosis, proliferation, CD31-positive vessels, and permeability in the neuroblastoma xenografts comparing vehicle-treated with ABT-510, VPA, and combination-treated groups. ANOVA followed by Dunnett’s multiple comparison procedure was used to compare the statistical significance of differences between control and treatment groups for sub-G1 and cell cycle analysis.

Results

VPA, but not ABT-510, inhibits neuroblastoma cell proliferation, blocks cell cycle progression, and induces apoptosis. To investigate the effects of VPA and ABT-510 as single agents on neuroblastoma cell growth in vitro, SMS-KCNR neuroblastoma cells were cultured in a range of drug concentrations. As shown in Supplementary Fig. S1, treatment with ABT-510 did not inhibit the proliferation of SMS-KCNR neuroblastoma cells even at concentrations as high as 1,000 nmol/L (Supplementary Fig. S1A and C). In contrast, VPA significantly inhibited neuroblastoma cell proliferation at concentrations of 5 and 10 nmol/L of VPA when the cells were cultured in medium containing either 10% or 2% serum (P < 0.01, respectively; Supplementary Fig. S1B and D). Furthermore, 2.5, 5.0, and 10 nmol/L of VPA induced neuroblastoma cell apoptosis as shown by the increased population of sub-G1 cells (P = 0.0014, P = 0.0008, and P < 0.0001, respectively; Fig. 1B), and cell cycle analysis revealed that treatment with VPA at concentrations of 2.5, 5.0, and 10 nmol/L induced G2 growth arrest (P = 0.0003, 0.0002, and 0.0004, respectively; Fig. 1C). Neither apoptosis (Fig. 1A) nor changes in cell cycle (Fig. 1C) were seen with ABT-510 (P > 0.01).

VPA and ABT-510 inhibit bFGF-induced endothelial cell migration. To test the antiangiogenic activity of VPA and ABT-510, endothelial cell migration assays were done using a range of drug concentrations in the presence or absence of bFGF. bFGF-induced endothelial cell migration was inhibited by ABT-510 and VPA in a dose-dependent manner at concentrations ranging from 0.1 to 100 nmol/L and 10 nmol/L to 1 mmol/L, respectively (Fig. 1D).

VPA down-regulates MYCN expression and induces TSP-1 and SPARC expression in neuroblastoma cells. Because MYCN plays a critical role in the regulation of neuroblastoma cell proliferation and apoptosis, we examined the levels of MYCN mRNA expression in neuroblastoma cells following treatment with 1 mmol/L VPA by quantitative real-time reverse transcription-PCR (RT-PCR). As shown in Supplementary Fig. S2A and B, MYCN expression was significantly down-regulated in both SMS-KCNR and NMB cell lines within 2 h of treatment. In contrast, MYCN expression did not significantly change following treatment with 100 nmol/L ABT-510 (data not shown). To determine if the antiangiogenic activity of VPA was due, at least in part, to up-regulation of endogenous inhibitors of angiogenesis in neuroblastoma cells, we also evaluated the level of expression of TSP-1 and SPARC by RT-PCR in the SMS-KCNR neuroblastoma cells following treatment with VPA. We found that treatment with 1 mmol/L VPA for 24 h resulted in up-regulation of the level of TSP-1 and SPARC expression (Supplementary Fig. S2C and D).

VPA and ABT-510 inhibit neuroblastoma xenograft growth. To examine the antitumor effects of VPA and ABT-510, nude mice with xenografts (~70 mm3 in size) established from two different MYCN-amplified neuroblastoma cell lines were treated with each agent alone and in combination. Tumor growth was inhibited with single-agent therapy, and enhanced effects were seen with combination therapy. After 20 days of treatment with VPA combined with ABT-510, tumor volume was reduced by 91% (P < 0.001; Fig. 2A) in the SMS-KCNR model and by 87% in the NMB xenografts (P = 0.029; Fig. 2B) compared with controls.

Because the effectiveness of some antiangiogenic strategies is inversely related to tumor burden, we repeated our studies in a set of animals with large xenografts that were ~380 mm3 in size. As
shown in Fig. 2C, control tumor volumes increased 3.5-fold over the subsequent 10 days, and tumors in animals treated with ABT-510 or VPA, respectively, showed 33% and 44% reduced growth increases ($P < 0.001$ and 0.001). However, during this interval, animals receiving combined treatment had total cessation of tumor volume increase from the start of treatment through the entire 10-day period. Thus, the combination of ABT-510 and VPA achieved immediate tumor growth stasis ($P < 0.001$), whereas the individual treatments gave only modest inhibition.

**Cell apoptosis is increased when VPA is combined with ABT-510.** To evaluate whether treatment induced apoptosis, TUNEL assays were done on the neuroblastoma xenografts treated with ABT-510, VPA, or combination therapy. The tumors treated with ABT-510 contained significantly more apoptotic cells than the control tumors (53 ± 16 versus 34 ± 9, $P = 0.015$; Fig. 3A). However, the highest number of apoptotic cells was seen in the xenografts treated with VPA alone or VPA combined with ABT-510 (93 ± 13 and 141 ± 39, respectively, $P < 0.001$).

**VPA and ABT-510 inhibit neuroblastoma tumor cell proliferation in vivo.** Cell proliferation in the xenografts was evaluated by Ki-67 expression, a nuclear protein that is preferentially expressed during active phases of the cell cycle. Ki-67–negative cells represent quiescent G0 phase cells. A significantly higher percentage of neuroblasts in G0 were detected in tumors treated with VPA alone compared with control tumors (50.6 ± 4.7 versus 34.2 ± 9, respectively, $P < 0.01$; Fig. 3B). The percentage of neuroblasts in G0 in the control tumors and ABT-510–treated tumors was not significantly different (34.2 ± 9 versus 42.4 ± 5.3, $P = 0.076$; Fig. 3B). However, the percentage of G0 cells was significantly increased in the tumors treated with combination therapy compared

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**Figure 3.** A, representative photographs of TUNEL fluorescent apoptosis in situ detection assay from tumors treated with VPA alone, ABT-510 alone, or ABT-510 and VPA in combination. Magnification, ×400. Mean apoptosis counts per 10 HPPFs in treated tumors versus controls. B, Ki-67 immunostained sections. Proliferating cells were identified by Ki-67 antibody in control versus ABT-510, VPA, and combination–treated groups. Percentage of G0 (Ki-67–negative cells) per 10 HPPFs of the control and treated tumors. C, MVD in SMS-KCNR xenografts after treatment with ABT-510, VPA, or ABT-510 and VPA in combination. Columns, mean MVD measured in SMS-KCNR xenografts following treatment with vehicle, ABT-510, VPA, or combination therapy; bars, SD. P < 0.001, vehicle versus VPA; P < 0.001, vehicle versus ABT-510; P < 0.001, vehicle versus combination.
with control tumors or tumors treated with either single agent (86.4 ± 9.1 versus 34.2 ± 9, P < 0.01; 86.4 ± 9.1 versus 42.4 ± 5.3, P < 0.01; 86.4 ± 9.1 versus 50.6 ± 4.7, P < 0.01; Fig. 3).

**Table 1. Effects of ABT-510 and VPA treatment on neuroblastoma tumor blood vessel morphology**

<table>
<thead>
<tr>
<th>Vehicle (% ± SE)</th>
<th>ABT-510 (% ± SE)</th>
<th>VPA (% ± SE)</th>
<th>ABT-510 + VPA (% ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. NMB xenografts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment started when tumors were &lt;70 mm³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEP (type 1)</td>
<td>58.00 ± 9.04</td>
<td>7.85 ± 5.35</td>
<td>12.69 ± 3.53</td>
</tr>
<tr>
<td>Thin-walled vessels</td>
<td>42.00 ± 9.04</td>
<td>92.15 ± 5.35</td>
<td>87.31 ± 5.53</td>
</tr>
<tr>
<td>Thin or capillary like (type 2)</td>
<td>69.05 ± 9.91</td>
<td>62.04 ± 7.11</td>
<td>68.55 ± 12.12</td>
</tr>
<tr>
<td>Embedded in fibrocollagenous stroma (type 3)</td>
<td>30.95 ± 9.91</td>
<td>37.96 ± 7.11</td>
<td>31.45 ± 12.12</td>
</tr>
<tr>
<td>Hyperplastic arteriolosclerosis (type 4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>B. SMS-KCN rat xenografts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment started when tumors were ≥380 mm³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEP (type 1)</td>
<td>100 (network)</td>
<td>14.68 ± 3.83</td>
<td>2.09 ± 0.72</td>
</tr>
<tr>
<td>Thin-walled vessels</td>
<td>0</td>
<td>83.32 ± 3.85</td>
<td>97.91 ± 0.72</td>
</tr>
<tr>
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<td>0</td>
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<td>21.33 ± 4.96</td>
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<tr>
<td>Embedded in fibrocollagenous stroma (type 3)</td>
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<td>80.05 ± 4.50</td>
<td>78.67 ± 4.96</td>
</tr>
<tr>
<td>Hyperplastic arteriolosclerosis (type 4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

**Note:** The values for thin-walled vessels were (type 2) and capillary vessels (type 3) were taken from Table 1A to Table 1B.

**Figure 4.** Representative sections from H&E-stained and Masson’s trichrome–stained control and treated NMB xenografts. Hemorrhage is moderate in control tumors, mild in ABT-510–treated group, minimal in VPA–treated group, and absent following combination treatment. Control tumors show predominantly VEP (type 1 vessels; middle). To investigate if the treatment with either agent alone or in combination was capable of inducing expression of this angiogenic inhibitor. No significant up-regulation of TSP-1 was observed in treated tumors.

**Treatment with ABT-510 and VPA significantly inhibits neuroblastoma tumor angiogenesis.** To investigate the antiangiogenic effects of ABT-510 and VPA, the vascularity of tumors treated early with ABT-510, VPA, or ABT-510 and VPA in combination was evaluated. Histologic sections were stained with CD31 to highlight endothelial cells, and a MVD was quantified. Compared with control tumors, a significantly lower MVD was seen in xenografts following treatment with VPA or ABT-510 (247,805 ± 73,062 versus 114,597 ± 47,568 versus 75,880 ± 31,638, respectively, P = 0.00024; Fig. 3C). The MVD was significantly lower in the tumors treated with combination therapy (247,805 ± 73,062 versus 31,406 ± 18,401; Fig. 3C) than in either single-agent group (P < 0.001). We also evaluated TSP-1 expression in the control and treated SMS-KCN rat xenografts using immunohistochemistry to determine if the treatment with either agent alone or in combination was capable of inducing expression of this angiogenic inhibitor. No significant up-regulation of TSP-1 was observed in treated tumors.

**VPA and ABT-510 treatment decreases hemorrhage and modifies tumor vessel structure.** To investigate the effects of VPA and ABT-510 on tumor growth, treatment was initiated in animals with tumors measuring ~70 mm³. Mice were sacrificed after 20 days of treatment, and the tumors were histologically examined. The control NMB neuroblastoma xenografts were grossly hemorrhagic, and extravasated RBCs were seen microscopically. The tumor blood vessels were morphologically classified into four types as described in Materials and Methods. More than half of the vessels in the control neuroblastoma xenografts were structurally abnormal with VEP (type 1 vessels; 58 ± 9.04% [Fig. 4, Control, left and right; Table 1A]). The remaining vessels were thin walled and capillary like (type 2; Fig. 4, Control, middle; Table 1A). To investigate if the structurally abnormal vessels were related to the large size of the control tumors, we repeated these experiments and sacrificed mice when the tumors were similar in size to the treated tumors (ranging from 220 to 726 mm³). Similar to the large tumors, significant hemorrhage was present in the smaller control tumors, and the majority of vessels were structurally abnormal and with VEP.

Tumor hemorrhage was decreased in experiments in which treatment with ABT-510 was started when tumors were ~70 mm³ in size. Significantly fewer type 1 blood vessels were observed (7.85 ± 5.35% [P = 0.0028] in these tumors, and the majority of the vessels were thin walled (type 2 and 3 vessels; Fig. 4, ABT-510, right, middle, and left; Table 1A). Virtually no hemorrhage was seen in tumors treated with VPA, and the percentage of blood vessels with VEP (type 1 vessels) decreased to 12.69 ± 3.5% (P = 0.0039). The majority of vessels in these tumors were thin walled and many were capillary like (Fig. 4, VPA, right, middle, and left; Table 1A). Some of the vessels were embedded in fibrocollagenous stroma, highlighted by Masson’s trichrome special stain (type 3 vessels), and others were arteriolar with mild hyaline sclerosis (Table 1A). In tumors treated with VPA combined with ABT-510, hemorrhage was not

Figure 5. Representative H&E-stained sections of SMS-KCNR xenografts treated at reaching ≥380 mm³ in size. Severe hemorrhage is seen in the control tumors (left and middle). Hemorrhage is moderate in the tumors treated with ABT-510 (left and middle), minimal in VPA-treated tumors (left and middle), and absent following combination treatment (left and middle). Control tumors show extensive VEP (type 1 vessels; middle and right). Tumors treated with ABT-510 show a few type 1 vessels (right) and a majority of thin-walled type 2 and 3 vessels (middle). In the VPA-treated tumors, most vessels are thin walled, including capillary like and arteriolar (type 2 vessels; middle) as well as those embedded in fibrocollagenous stroma (type 3 vessels; right). Tumors from the ABT-510 + VPA combination-treated group show thin-walled vessels surrounded by fibrosis (middle). A subset of these tumors showed small vessel changes with features of hyperplastic arteriolosclerosis (type 4 vessels; right). Arrows, vessels.

seen and the proportion of vessels with VEP was further reduced to 3.55 ± 1.12% (P = 0.0007; Table 1A). The vessels in the tumors treated with combination therapy were thin walled and embedded in fibrocollagenous stroma (type 3; Fig. 4, ABT-510 + VPA, left and right) or capillary like (type 2; Fig. 4, ABT-510 + VPA, middle).

We also evaluated the effects of ABT-510 and VPA on the growth of large established neuroblastoma tumors. Similar to the previous experiment, significant hemorrhage was seen in the control SMS-KCNR tumors that were ≥380 mm³ in size when treatment was started (Fig. 5, Control, left and middle). These tumors had a continuous network of structurally abnormal type 1 vessels (Fig. 5, Control, right, middle, and left; Table 1B). Following treatment with ABT-510, the tumors were less hemorrhagic than the controls (Fig. 5, ABT-510, left and middle; Table 1B) and type 1 vessels constituted only 14.68 ± 3.83% (P < 0.0001) of the total (Fig. 5, ABT-510, right). The majority of vessels were thin walled, and some were embedded in fibrocollagenous stroma (Fig. 5, ABT-510, middle). In VPA-treated tumors, hemorrhage was minimal (Fig. 5, VPA, left and middle) and the number of the type 1 vessels was only 2.09 ± 0.72% (P < 0.0001; Table 1B). Almost all of the vessels in the VPA-treated tumors were thin walled (Fig. 5, VPA, middle and right; Table 1B). Some vessels were capillary like (Fig. 5, VPA, middle), some had mild hyaline sclerosis (Fig. 5, VPA, middle), and others were embedded in fibrocollagenous stroma (Fig. 5, VPA, right; Table 1B). Minimal hemorrhage was seen in the tumors treated with both VPA and ABT-510 (Fig. 5, ABT-510 + VPA, left and middle), and VEP was absent. The majority of vessels in these tumors were thin walled and classified as type 2 or 3 vessels (Fig. 5, ABT-510 + VPA, middle; Table 1B). In three of the seven tumors treated with combination therapy, there were small numbers of vessels with thickened walls exhibiting concentric lamellar cellular arrangement (‘onionskin’ pattern) with marked luminal narrowing, focal fibrosis, fibrinoid necrosis, and vasculitis (type 4 vessels, hyperplastic arteriolosclerosis; Fig. 5, ABT-510 + VPA, right; Table 1B).

VPA and ABT-510 treatment decreases vascular permeability.

Figure 6. Evan’s blue dye assay for intratumoral vessel permeability of the SMS-KCNR xenografts. Quantitative analysis shows reduction of Evan’s blue dye extravasation in mice treated with ABT-510, VPA, and ABT-510 and VPA in combination compared with controls. RQ, relative quantification by spectrophotometry.

Tumor blood vessels are functionally abnormal and leaky. To determine if treatment with VPA and ABT-510 modified vessel function, we assessed intratumoral vessel permeability using the Evan’s blue dye assay. In this assay, Evan’s blue dye is given systemically, mice are then perfused with saline to wash out the intravascular dye, and the amount of Evan’s blue dye remaining in the tumor correlates to permeability of the microvasculature. Our results indicate a decrease in intratumor vessel permeability in all treatment groups (Fig. 6). The decrease was modest in the tumors treated with ABT-510 alone compared with controls and did not reach statistical significance (P = 0.124; Fig. 6). However, the reduction in vascular permeability was statistically significant in tumors treated with either VPA alone or in combination with ABT-510 compared with controls (P = 0.044 and 0.007, respectively; Fig. 6).

Discussion

More than 30 years ago, Folkman (33) proposed the concept that blocking angiogenesis would result in tumor dormancy. Clinical trials in adults with cancer have supported this hypothesis, and the antiangiogenic agent bevacizumab (Avastin)
was recently granted Food and Drug Administration approval for use in metastatic colon cancer (34). ABT-510, HDAC inhibitors, and other promising antiangiogenic agents, either alone or in combination with cytotoxic agents, are currently being tested in adult clinical trials. Although we and others have reported that the growth of common pediatric cancers, such as neuroblastoma and Wilms’ tumor, is also angiogenesis dependent (4, 35), to date, the experience with antiangiogenic agents in childhood malignancies is limited. In this study, we evaluated the effects of ABT-510 and VPA on neuroblastoma angiogenesis and tumor growth. As expected, both agents blocked bFGF-induced endothelial cell migration in vitro. In addition to inhibiting angiogenesis, VPA also inhibited neuroblastoma cell proliferation and induced neuroblastoma apoptosis. Furthermore, MYCN expression levels were significantly down-regulated by VPA, suggesting that this agent is capable of affecting the biology of neuroblastoma cells. Similar changes in MYCN expression occur in neuroblastoma following treatment with retinoic acid, a drug that is of clinical benefit in children with high-risk neuroblastoma (36). We also found that VPA induced the expression of endogenous inhibitors of angiogenesis, including TSP-1 and SPARC. Previous work from our laboratory has shown that SPARC is a potent inhibitor of angiogenesis in neuroblastoma (27). In contrast, ABT-510 had no effect on the neuroblastoma cell growth in vitro. As single agents, both ABT-510 and VPA inhibited neuroblastoma tumor growth in vivo, and enhanced antitumor effects were seen with combination therapy.

ABT-510 has been extensively studied in cancers that are common in adults (15), but to our knowledge, this is the first report showing that this agent has antiangiogenic activity in the pediatric cancer neuroblastoma. TSP-1 and ABT-510 act by inducing endothelial cell apoptosis, and recent studies suggest that TSP-1 generates CD95L, a ligand for the CD95 death receptor (37). However, because CD95 expression on the vascular endothelial cell is independent of TSP-1, the efficacy of the drug is limited. In an effort to develop a more effective antiangiogenic treatment strategy, ABT-510 has been combined with other agents in preclinical models of adult cancer (38). We selected VPA for our combination studies for several reasons. First, VPA is a potent inhibitor of angiogenesis. Although its mechanism of action remains unclear, VPA has been shown to relieve HDAC-dependent transcription repression of several antiangiogenic proteins (39). Second, VPA directly affects the growth of neuroblastoma cells by inhibiting cell cycle progression and inducing differentiation (16). Third, VPA has been shown to have synergistic effects with other drugs that regulate angiogenesis, including IFN-α and retinoic acid (17, 18). Finally, VPA has limited toxicities even during long-term treatment. Other HDAC inhibitors have also been shown to inhibit neuroblastoma tumor growth. The hybrid polar HDAC inhibitor m-carboxyoxynamic acid bis-hydroxamide (CBHA) induces apoptosis in neuroblastoma cells in vitro and suppresses the growth of neuroblastoma xenografts. The combination of CBHA and all-trans retinoic acid led to an even greater inhibition of tumor growth (40). Jaboin et al. (41) reported that the synthetic benzamide derivative MS-27-275 (MS-275) inhibited [3H]thymidine uptake in a series of pediatric tumor cell lines and suppressed the growth of undifferentiated sarcoma, Ewing’s sarcoma, and neuroblastoma in vivo. These investigators reported a marked decrease in the vascularization of neuroblastoma xenografts following treatment with MS-275. Others (42) have indicated that trichostatin A (TSA) and suberoylanilide hydroxamic acid can block angiogenesis and that TSA specifically inhibits hypoxia-induced angiogenesis in the Lewis lung carcinoma model.

In the experiments done on both small and large neuroblastoma xenografts, the combination of ABT-510 and VPA immediately and fully stabilized the size of the tumors for 20 and 10 days, respectively, in the NMB (early treatment) and SMS-KCNR (late treatment) models during which time vehicle controls substantially increased in size. The MVD was significantly lower in xenografts treated with combination therapy compared with tumors treated with single agents or controls. Furthermore, ABT-510 combined with VPA induced significant apoptosis and inhibited neuroblastoma cell proliferation. Previous studies done in our laboratory with TNP-470 showed that this antiangiogenic agent was capable of inhibiting the growth of small neuroblastoma xenografts but did not affect the rate of growth of large neuroblastoma tumors (5). In contrast, we found that the combination of ABT-510 and VPA was also capable of stabilizing the growth of large xenografts.

Hemorrhage was reduced in the neuroblastoma xenografts treated with single-agent therapy compared with control tumors, and virtually no hemorrhage was seen in the tumors treated with combination therapy. In addition, striking changes in blood vessel morphology were seen following treatment with ABT-510 and VPA alone or in combination. Control tumors contained large numbers of structurally abnormal blood vessels with VEP. VEP is an established unfavorable factor in several cancers, including melanoma, breast, prostate carcinomas, and glioblastoma (43, 44), and it is one of the criteria for raising the WHO grade of astrocytic neoplasms (45). The number of vessels with VEP was significantly decreased in the treated tumors, whereas the proportion of thin-walled, capillary-like vessels was increased. Some of the thin-walled vessels in the treated tumors were embedded in fibrocollagenous stroma, whereas others exhibited hyaline sclerosis, a feature that is usually seen in hypertensive disease and attributed to endothelial cell injury (46–48). In the large SMS-KCNR tumors treated with combination therapy, rare vessels with hyperplastic arteriolosclerosis were also seen. Using the Evan’s blue dye assay, we were also able to show that treatment with ABT-510 and VPA resulted in modification of vessel function with decreased permeability of the tumor vasculature.

Our results indicate that, in addition to reducing the number of blood vessels, the combination of ABT-510 and VPA modifies the structure and function of the vasculature in neuroblastoma tumors. Transient “normalization” of tumor vasculature following treatment with other antiangiogenic agents has recently been reported (25). These changes create a vasculature that is more efficient for oxygen and drug delivery and may thereby lead to chemosensitization (34). Thus, in addition to inhibiting neuroblastoma tumor growth directly, it is possible that ABT-510 and VPA may also enhance the antineuroblastoma activity of cytotoxic agents. Additional studies evaluating the clinical efficacy of this antiangiogenic treatment strategy in children with high-risk neuroblastoma are warranted.

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**Valproic Acid and TSP-1 Inhibit Neuroblastoma Growth**


References


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