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HIF-1 α activation mediates resistance to anti-angiogenic therapy in neuroblastoma xenografts

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Abstract

Introduction—The anti-tumor activity of angiogenesis inhibitors is often limited by the development of resistance to these drugs. Here we establish HIF-1 α as a major factor in the development of this resistance in neuroblastoma xenografts.

Methods—Neuroblastoma xenografts were established by injecting unmodified SKNAS or NB-1691 cells (2×10^6 cells), or cells in which HIF-1 α expression had been knocked down with shRNA, into the retroperitoneal space of SCID mice. Treatment of established tumors included bevacizumab (5mg/kg q2wk), sunitinib (40mg/kg qd), or topotecan (0.5mg/kg qd) alone or in combination for a total of two weeks.

Results—NB-1691 xenografts showed no difference in relative growth in HIF-1 α knockdowns compared to control tumors (73.33 ± 7.90 vs 79.94 ± 6.15 , $p=0.528$). However, HIF-1 α knockdowns demonstrated relative final volumes that were significantly lower than unmodified tumors when both were treated with bevacizumab (35.88 ± 4.24 vs 53.57 ± 6.61 , $p=0.0544$) or sunitinib (12.46 ± 2.59 vs 36.36 ± 4.82 , $p=0.0024$). Monotherapy of unmodified xenografts with bevacizumab, sunitinib, or topotecan was largely ineffective. Relative final volumes of NB-1691 xenografts were significantly less in cohorts treated with sunitinib+topotecan (4.78 ± 0.77 vs 39.17 ± 2.44 [sunitinib alone], $p=0.011$) and bevacizumab+topotecan (13.63 ± 1.55 vs 48.16 ± 9.94 [bevacizumab alone], $p=0.014$).

Conclusion—Upregulation of HIF-1 α appears to be a significant mechanism of resistance to antiangiogenic therapies in neuroblastoma. Suppressing HIF-1 α with low-dose topotecan potentiates the effects of the antiangiogenic drugs in a mouse model.

Keywords

Bevacizumab; Sunitinib; Angiogenesis; HIF-1 α ; Neuroblastoma

Neuroblastoma is the most common extracranial solid tumor in children. Despite extensive research and novel treatments, the survival for advanced stage neuroblastoma patients remains poor with 5 year survival of stage 4 patients of 30%–40% [1].

The idea of attacking tumor vessels as a form of cancer therapy was first proposed by Dr. Judah Folkman in the late 1960s. Over thirty years later, Avastin[®] (bevacizumab,

Genentech) became the first angiogenesis inhibitor approved by the FDA. Despite demonstrated pre-clinical efficacy, the use of bevacizumab as monotherapy has been largely ineffective in clinical trials [2]. A second generation of angiogenesis inhibitors has also been approved. These drugs, termed collectively receptor tyrosine kinase inhibitors, (RTKI) have shown some promise as monotherapy in specific cancers (RCC and GIST) but have not demonstrated single agent efficacy in neuroblastoma [3]. Despite a somewhat disappointing response to monotherapy, both treatments have demonstrated significant improvements when added to standard chemotherapeutic regimens [4]. The poor response to monotherapy is likely due to a multitude of resistance mechanisms that occur both in intracellular and extracellular environments in treated tumors. These mechanisms include the up-regulation of VEGF and VEGFR, alternative angiogenic pathways such as EGF and PDGF, and up-regulation of the hypoxia inducible factor (HIF) and its associated gene products [5]. Overcoming this resistance is essential for effective treatment using angiogenesis inhibitors. We chose to focus specifically on the up-regulation of HIF-1 α as a mechanism of resistance to angiogenesis inhibitor treatment.

HIF-1 α is a heterodimeric protein composed of two subunits. The HIF-1 α subunit is constitutively expressed but in the presence of oxygen is rapidly bound to the von Hippel-Lindau (VHL) tumor suppressor, ubiquitinated, and targeted for proteasomal degradation. In the absence of oxygen, HIF-1 α binds to the HIF-1 β subunit and co-localizes to the nucleus where it activates transcription of a host of genes whose functions include angiogenesis, glycolysis, migration, and metastasis. Immunohistochemical staining of biopsies of tumors from neuroblastoma patients reveals up-regulation of HIF-1 α and HIF-2 α , a similar factor with a similar effect [6]. The rapid up-regulation of HIF-1 α creates a “super tumor cell” which is capable of surviving in otherwise hostile environments, is largely resistant to anti-angiogenic and conventional chemotherapeutic treatments and is more likely to metastasize.

Normal cells grow on a gradient of oxygen diffusion, whereby cells growing closest to vessels have the highest intracellular oxygen concentration and oxygen concentrations decrease in cells farther away from vessels. Within the solid tumor microenvironment, this gradient is quite steep with areas of the tumor distant from functioning vessels undergoing necrosis due to hypoxia. This leads to areas of increased hypoxia in some parts of the tumor while actually shunting blood and improving perfusion to other parts of the tumor [8]. Within this flux of oxygen delivery to tumor cells, those on the periphery of diffusion are likely to up-regulate HIF-1 α . This in turn not only allows the cells to survive in a “hostile” environment by activating genes for angiogenesis and glycolysis, but also activates genes which increase migration and metastasis. This may have some role in the incidence of increased metastasis seen in pre-clinical models following anti-angiogenesis treatment [7].

Topotecan at low doses has been shown to inhibit the up-regulation of HIF-1 α and HIF-2 α in neuroblastoma cells in vitro at less than cytotoxic doses [8]. Topotecan is a camptothecin inhibitor of topoisomerase I. It is frequently used at maximum tolerated doses (MTD) for the treatment of many solid tumors, including neuroblastoma. At MTD topotecan is known to have a multitude of toxic side effects. However, topotecan is one of several chemotherapeutic drugs that have been shown to inhibit HIF-1 α expression at less than MTD. At lower doses, one can achieve the desired effect of inhibition of HIF-1 α without the toxic side effects. Low dose topotecan has been used to potentiate the effects of bevacizumab in subcutaneous glioblastoma xenografts [9]. Similarly, shRNA targeted against the HIF-1 α message has been used in vitro in neuroblastoma cells to downregulate HIF-1 α expression making cells susceptible to hypoxic damage [10].

In order to assess the contribution of HIF-1 α activation to anti-angiogenic resistance, we sought to study the effects of two well-known anti-angiogenic drugs, bevacizumab and

sunitinib (Sutent[®], Pfizer) in neuroblastoma xenografts. We studied both the in vitro and in vivo effects of blocking HIF-1 α by using low dose topotecan and developing HIF-1 α knockdown mutants using shRNA targeted against the HIF-1 α gene product. We hypothesized that the inhibition of HIF-1 α would eliminate a major resistance mechanism to anti-angiogenic therapy, thereby potentiating the treatment effects of bevacizumab and sunitinib. The goal of these studies was to test the efficacy of the co-administration of an anti-angiogenic agent and HIF-inhibitor in a relevant pre-clinical model as a potential therapeutic approach that might then be tested in clinical trials for treating children with neuroblastoma.

1. Methods

1.1. Cell Lines

The human neuroblastoma cell line, NB1691, was provided by P. Houghton (Columbus, OH). The CHLA-20 cell line was provided by C.P. Reynolds (Los Angeles, CA). SKNAS was obtained from ATCC (Manassas, VA).

1.2. Generation of shHIF-1 α Neuroblastoma Cells

Exponentially growing SKNAS and NB1691 neuroblastoma cells were plated into 24-well tissue culture plates at 5×10^4 cells/well. The next day, the cells were transfected with shHIF-1 α plasmids containing the puromycin resistance gene (SABiosciences, Valencia, CA) with Fugene HD (Roche Applied Science, Indianapolis, IN) as the transfection reagent. Western blots were performed using an anti-HIF-1 α antibody (BD Biosciences, San Jose, CA) to confirm HIF-1 α knockdown.

1.3. Murine orthotopic models

All animal studies were approved by institutional IACUC prior to initiation. Tumor xenografts were first established by injecting 1×10^6 shHIF-1 α modified NB1691 or SKNAS cells into the retroperitoneal space of CB-17 SCID mice (Taconic Farms, Hudson NY). One group was injected with HIF-1 α knocked down cells and a second control group was injected with mock transfected cells (control). Ten days after injections, all retroperitoneal tumors were size matched using three dimensional ultrasound (GE, New York, NY). Following this, three separate groups of five mice each received one of the following treatments; control (vehicle only), bevacizumab (5mg/kg daily, intraperitoneal), or sunitinib (40mg/kg daily, oral gavage). After two weeks of treatment, the tumors were again evaluated by ultrasound. Tumor volumes were reported as relative volume by dividing final tumor volume by initial tumor volume. All statistics were analyzed using Prism (GraphPad Software, La Jolla, CA). All results are reported as mean $\pm$ SEM unless otherwise stated.

Retroperitoneal tumors were then again established in CB-17 mice as described above using unmodified CHLA-20 and NB1691 cell lines. After two weeks, tumor bearing mice were imaged via ultrasound and the tumors were size matched into 6 groups of five mice each. For each of the cell lines, the groups received the following treatments; control (vehicle only), bevacizumab (5mg/kg daily, intraperitoneal), sunitinib (40mg/kg daily, oral gavage), topotecan (0.5mg/kg daily, intraperitoneal), or combinations of bevacizumab/topotecan (5 mg/kg+0.5mg/kg daily intraperitoneal) or sunitinib/topotecan (40mg/kg+0.5mg/kg daily oral gavage and intraperitoneal respectively) for two weeks. At the end of 2 weeks, the tumors were re-imaged to determine tumor volume, blood was collected, and the mice were sacrificed. Triplicate sera samples from the mice were used to detect human VEGF-A and mouse VEGF levels by ELISA (Raybiotech, Norcross GA). A portion of the retroperitoneal tumor was snap frozen in liquid nitrogen and an additional portion was preserved in formalin.

1.4. RT-PCR of HIF-1 α dependent genes

Total RNA was extracted from tumor samples according to the manufacturer's instructions (Tel-Test, Friendswood, TX). The RNA was reversed transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and the cDNA was amplified using primers against VEGFA (Hs00173626_m1, Applied Biosystems, Foster City, CA) and GLUT3 (Roche Applied Science, Indianapolis, IN), both HIF1- α dependent factors.

1.5. CD34 Staining of Tumor Samples

Retroperitoneal tumors were fixed in formalin, paraffin embedded, and sectioned. The slides were stained for CD34 as previously described [7]. Sections were viewed and digitally photographed using a Nikon Eclipse E400 light microscope with an attached camera. Four images were taken of each tumor section with care to avoid areas of necrosis. Images were saved as JPEG files for further processing in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA). Positive staining was quantified using an NIH image analysis software (ImageJ) and is reported as the mean number of positive pixels per tumor section.

2. Results

2.1. Neuroblastoma tumors with shHIF-1 α inhibition demonstrate increased sensitivity to anti-angiogenesis drugs in mice

Tumors formed from neuroblastoma cells with shHIF-1 α were compared to control tumors that were mock-transfected. For the NB1691 group, there was no significant difference between the shHIF-1 α and mock transfected groups (69.3 ± 9.2 vs. 79.9 ± 6.1 , $p=0.484$) (Fig. 1A). In the bevacizumab treated group, the rate of growth of the HIF-1 α knockdowns was approximately one third of the mock transfected control group (39.9 ± 6.0 vs 51.6 ± 7.4 , $p=0.41$). The HIF-1 α knockdowns treated with sunitinib had a more profound and significant decrease ($>60\%$) in tumor volume compared to the mock transfected controls (12.5 ± 2.6 vs 36.4 ± 4.8 , $p=0.0024$). A similar effect was also observed in tumors from SKNAS cells transfected with shHIF-1 α (Fig. 1A). The inhibition of HIF-1 α decreased the growth of bevacizumab treated tumors by 20% (30.5 ± 2.5 vs. 37.8 ± 2.9 , $p=0.165$). The tumor volumes in the sunitinib cohort had a $>50\%$ inhibition when compared to mock transfected controls (16.1 ± 1.9 vs. 34.7 ± 2.3 , $p=0.004$). These experiments demonstrate that inhibition of HIF-1 α augmented the effects of anti-angiogenesis treatment in a mouse model of neuroblastoma, establishing HIF1- α upregulation as a major mechanism of resistance.

2.2. The addition of low dose topotecan to the angiogenesis inhibitors bevacizumab and sunitinib results in dramatically decreased tumor growth

Retroperitoneal tumors were established and treated as indicated in the methods section. Tumor volumes were measured by ultrasound and reported as relative tumor growth (final volume/initial volume). For the NB1691 cell line, there was no significant difference between the bevacizumab only, topotecan only and control groups (48.2 ± 9.9 , 38.0 ± 10.3 vs. 54.1 ± 2.3 , $p=0.583$) (Fig. 1B). However, there was a difference between the bevacizumab/topotecan combination group compared to bevacizumab treatment alone (13.6 ± 1.5 vs. 48.2 ± 9.9 , $p=0.014$). There was also a significant difference in the sunitinib group alone compared to control (39.1 ± 2.4 vs 54.1 ± 2.35 , $p=0.0035$). This difference was greatly enhanced with the addition of topotecan to sunitinib compared to sunitinib alone (4.8 ± 0.8 vs 39.1 ± 2.4 , $p=0.0001$). These experiments were repeated with a different neuroblastoma cell line (CHLA-20) with similar results (Fig. 1B). Like the NB1691 tumors, the bevacizumab/topotecan (17.4 ± 2.2 , $p=0.049$) and sunitinib/topotecan (12.8 ± 1.3 , $p=0.028$) groups had significantly smaller tumors compared to either bevacizumab (40.06 ± 8.22) or sunitinib (31.16 ± 6.72) given singly.

2.3. Serum levels of human and mouse VEGF are decreased in tumors treated with HIF1- α inhibition

Serum was collected from all animals in the above experiment at the time of sacrifice. The level of human VEGF was evaluated by ELISA. This ELISA is specific for human VEGF and will detect VEGF secretion only from the tumor xenografts and not the mouse derived stromal tissues. In NB1691 tumors, human VEGF levels in the bevacizumab and sunitinib only group were not significantly different from control (89.3 ± 13.9 pg/ml vs 97.0 ± 19.4 pg/ml, $p=0.75$ and 138.9 ± 29.1 pg/ml vs 97.0 ± 19.4 pg/ml, $p=0.258$ respectively). The addition of topotecan to either bevacizumab or sunitinib treatment significantly decreased the levels of human VEGF compared to bevacizumab (29.6 ± 2.3 pg/ml vs. 89.3 ± 13.9 pg/ml, $p=0.0006$) and sunitinib (39.5 ± 6.17 pg/ml vs 138.9 ± 29.1 pg/ml, $p=0.0075$) alone (Fig. 2A). This experiment was repeated with CHLA-20 tumors with similar results. Topotecan in combination with either bevacizumab or sunitinib decreased levels of VEGF compared to bevacizumab (25.5 ± 7.2 pg/ml vs 93.4 ± 24.5 pg/ml, $p=0.024$) and sunitinib (34.4 ± 11.5 pg/ml vs 217.6 ± 4.6 pg/ml, $p=0.001$) alone. This suggests that in tumors treated with anti-angiogenic drugs alone, up-regulation of VEGF is an escape mechanism that may lead to tumor resistance to therapy. We have shown that this mechanism can be blocked by the addition of topotecan. As expected, mouse VEGF levels were largely unaffected by treatment with bevacizumab. In NB1691 tumors, there was a dramatic increase in circulating mouse VEGF levels with monotherapy with bevacizumab (230 ± 38 pg/ml vs 59 ± 8 pg/ml, $p=0.005$) and sunitinib (290 ± 37 pg/ml vs 59 ± 8 pg/ml, $p=0.013$). The addition of topotecan decreased levels of mouse VEGF in both the bevacizumab/topotecan (73 ± 6.0 pg/ml vs. 227 ± 38 pg/ml, $p=0.0195$) and the sunitinib/topotecan (77 ± 4 pg/ml vs 288 ± 37 pg/ml, $p=0.002$) groups when compared to monotherapy (Fig. 2B). Experiments with CHLA-20 tumors gave similar results. Topotecan blocked a compensatory increase in mouse VEGF in the bevacizumab/topotecan (50.5 ± 4.5 pg/ml vs 143.0 ± 16.01 pg/ml, $p=0.0019$) and sunitinib/topotecan (55.1 ± 5.1 pg/ml vs 192.8 ± 52.4 , $p=0.026$) groups compared to monotherapy with either agent. The upregulation of mouse VEGF in tumors when treated with either bevacizumab or sunitinib alone demonstrates that signaling in focally hypoxic areas extends to nearby extra-tumoral stromal cells. Addition of topotecan dramatically decreased the circulating levels of mouse VEGF by blocking the local expression of HIF-1 α related factors.

2.4. RT-PCR detects changes in growth factors downstream of HIF1- α in treated tumors

HIF-1 α acts as a transcription factor for pro-angiogenic and glycolytic enzymes. An increase in VEGF due to an upregulation in HIF-1 α would correspond to an increase in VEGF transcription. Because HIF-1 α itself is relatively labile and transiently present in harvested tumors, we used RT-PCR to detect VEGF and GLUT3, which are up-regulated by HIF1- α . Although VEGF up-regulation is dependent on HIF1- α , it can also be up-regulated by other responses to local tumor hypoxia and tumor necrosis (NO, TNF- α). GLUT3 is a glucose transporter up-regulated by HIF-1 α only, it would not be directly affected by anti-angiogenic therapy [11]. Tumor samples from treated mice were prepared as indicated in the Methods section. The HIF1- α dependent growth factors, VEGF and GLUT3 were increased when tumors were treated with bevacizumab and sunitinib.

In NB1691 tumors treated with bevacizumab only, a two-fold increase in VEGF was detected compared to control (2.3 ± 0.324 vs 0.91 ± 0.058 , $p=0.0093$). The addition of topotecan decreased the level to approximately the same as control (0.937 ± 0.233 vs 0.91 ± 0.058 , $p=0.9302$) (Fig. 3A). Similar results were obtained in the sunitinib treated tumors. Treatment with sunitinib increased VEGF two-fold compared to control (2.13 ± 0.183 vs 0.91 ± 0.058 , $p=0.001$), but this effect was abrogated with the addition of topotecan (0.425 ± 0.033 vs 0.91 ± 0.058 , $p=0.0004$). These results were similar for the CHLA-20

tumors. Tumors treated with bevacizumab alone demonstrated a two-fold increase in VEGF compared to control (2.4 ± 0.145 vs 1.14 ± 0.03 , $p = 0.0001$). The addition of topotecan decreased the level to approximately the same as control (1.27 ± 0.037 vs 1.14 ± 0.03 , $p = 0.0558$). Treatment with sunitinib increased VEGF mRNA two-fold compared to control, but this effect was abrogated with the addition of topotecan (2.09 ± 0.349 vs. 0.687 ± 0.033 , $p = 0.0067$).

A similar effect was also seen in GLUT3. Bevacizumab therapy alone increased GLUT3 levels to 1.5 times higher than control (2.01 ± 0.20 vs. 1.04 ± 0.02 , $p = 0.019$). Once again, this effect was abolished by the addition of topotecan and levels were similar to control (1.46 ± 0.09 vs. 1.04 ± 0.02 , $p = 0.07$) (Fig. 3B). In the sunitinib treatment group, GLUT3 expression was increased two-fold and addition of topotecan decreased levels to baseline (1.92 ± 0.08 vs. 1.12 ± 0.05 , $p = 0.001$). The NB1691 tumors did not express GLUT3, and were not included in this analysis. Monotherapy with anti-angiogenic drugs up-regulate VEGF in both NB1691 and CHLA-20 tumors and GLUT3 in CHLA-20, likely through adaptation to the hypoxic tumor microenvironment. Blocking HIF-1 α with low dose topotecan appears to attenuate this effect, directly implicating HIF-1 α as a major resistance mechanism to anti-angiogenic therapy.

2.5. Topotecan in combination with bevacizumab or sunitinib decreases CD34+ cells in tumors

Previous studies have demonstrated that treatment with anti-angiogenic therapies reduces the number of CD34+ endothelial cells in tumors [7]. Additional research has demonstrated that this effect is temporal and CD34+ cells eventually increase as the tumors develop resistance to anti-angiogenic monotherapy [12]. In CHLA-20 tumors treated with bevacizumab and sunitinib alone, CD34+ cells were decreased (1060 ± 1700 , $p = 0.072$ and 2200 ± 360 , $p = 0.004$ respectively), compared to controls ($17,300 \pm 2600$) (Fig. 4A). In the bevacizumab/topotecan group, there was a more dramatic decrease in CD34+ cells compared to topotecan alone (5200 ± 470 vs. $10,600 \pm 1700$, $p = 0.004$) (Fig. 4B). The addition of topotecan to sunitinib treatment also decreased endothelial cells (1650 ± 420 vs. 2200 ± 3610 , $p = 0.378$), however these results were not statistically significant. This is likely due to a combination of a small sample size ($n = 5$) and the effectiveness of sunitinib as a single agent to curb endothelial cells proliferation. Overall, topotecan in combination with either bevacizumab or sunitinib resulted in decreased endothelial cell number within the tumor.

3. Discussion

Anti-angiogenic therapies represent a major breakthrough in cancer therapy due to their potentially profound effects on tumors with relatively low toxicity to the host. However, resistance to anti-angiogenic therapy remains a persistent problem which prevents the widespread use of these agents as monotherapy. There are multiple mechanisms of resistance involving a myriad of pathways. One that is of interest is the HIF-1 α signaling pathway because of the important role it plays in cancer pathophysiology and the ability of multiple agents to inhibit HIF production. We propose that within the areas of hypoxia found throughout a tumor, the HIF-1 α pathway is activated, which leads to the transcription of factors which promote angiogenesis, metastasis and chemoresistance. Therefore the inhibition of HIF-1 α is a key factor in preventing resistance to angiogenesis inhibitors. We sought to build on the work of previous researchers [8] by using low doses of topotecan to inhibit HIF-1 α , thereby abrogating this mechanism of resistance. Using topotecan in combination with either bevacizumab or sunitinib improved survival in mice with large retroperitoneal tumors.

Mice with shHIF-1 α tumors treated with either bevacizumab or sunitinib also showed a similar decrease in tumor size to wild type neuroblastoma tumors treated with topotecan in combination with either bevacizumab or sunitinib. This demonstrates that topotecan at low doses exerts its primary effects via HIF-1 α inhibition and not by increased cytotoxicity.

In these studies, VEGF was used to detect the presence of HIF-1 α up-regulation and tumor resistance to anti-angiogenic therapy. Treatment with anti-angiogenic agents as a monotherapy increased circulating VEGF levels as tumor cells within the hypoxic areas secrete more VEGF in response to HIF-1 α up-regulation; this effect was blocked by co-administration of low dose topotecan. It should be noted that both human VEGF (secreted by the tumor xenograft) and mouse VEGF (secreted by the host stroma) showed marked increase when tumor-bearing mice were treated with bevacizumab and sunitinib. This suggests that HIF-1 α up-regulation involves not only the tumor cells but also the surrounding host stroma. In addition, CD34 staining revealed a decrease in the number of endothelial cells in tumors treated with either bevacizumab or sunitinib in combination with topotecan compared to monotherapy alone although the sunitinib/topotecan combination did not reach statistical significance, which may be due to tumor sampling error.

We demonstrated changes in HIF-1 α activity by describing changes in both VEGF and GLUT3, which are factors known to be HIF-1 α dependent. While treatment with anti-angiogenic agents dramatically up-regulated these factors, this was blocked by the addition of low dose topotecan resulting in HIF-1 α inhibition.

Angiogenesis inhibitors provide a very promising target for cancer therapy. Targeting aberrant cancer cell physiology allows for destruction of tumor cells with minimal systemic toxicity. Although more advanced drugs continue to be developed, at present, resistance mechanisms appear to have limited the efficacy of this line of therapy. One mechanism of resistance is HIF-1 α activation within the tumor microenvironment. By activating HIF1- α , tumor cells are able to survive in a low oxygen environment and may be more likely to metastasize. Several chemotherapeutic agents including topotecan have been shown to limit HIF1- α expression in tumor cells, blocking this mechanism of resistance. Specific to neuroblastoma patients, bevacizumab and sunitinib are well tolerated with minimal side effects. Similarly, low dose topotecan is tolerated with minimal side effects and is available as an oral medication. It is feasible that this therapeutic combination may be given to patients with suspected minimal residual disease or unresectable disease in an outpatient setting. This study demonstrated the rationale for the addition of an agent that blocks HIF-1 α up-regulation in a clinical trial involving angiogenesis inhibitors in neuroblastoma. Further studies with more advanced angiogenesis inhibitors and additional combinations to block resistance are certainly warranted in the near future.

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Fig. 1.

The effects of anti-angiogenic therapy on modified and unmodified neuroblastoma cells. (A) NB1691 and SKNAS shHIF-1 α tumors treated with bevacizumab or sunitinib showed a decrease in tumor volumes compared to control. The decrease was significant in NB1691 and SKNAS tumors treated with sunitinib (* $p=0.0024$) and (** $p=0.004$) respectively. (B) Tumor volumes in single agent therapy of unmodified NB1691 and CHLA-20 neuroblastomas were not statistically different. Tumor volumes showed a dramatic decrease when topotecan was added to either bevacizumab or sunitinib regimen. (* $p=0.014$, ** $p=0.0035$, *** $p=0.049$, **** $p=0.028$). (C) Representative ultrasound image of tumor xenograft including kidney (green), tumor (red) and adrenal (purple).

Fig. 2.

Low dose topotecan blocks HIF-1 α mediated increases in VEGF after anti-angiogenic therapy. (A) Sera from mice implanted with NB1691 and CHLA-20 xenografts show an increase in human VEGF with sunitinib treatment but not bevacizumab (* $p=0.0075$, ** $p=0.024$, *** $p=0.001$). Topotecan effectively blocks this effect. (B) Mouse VEGF is increased with bevacizumab and sunitinib treatment. As with human VEGF, this effect was blocked with the topotecan (* $p=0.02$, ** $p=0.002$, *** $p=0.002$, **** $p=0.026$).

Fig. 3.

RT-PCR of human VEGF and GLUT3. (A) NB1691 and CHLA-20 neuroblastoma treated for two weeks with bevacizumab or sunitinib had increased levels of VEGF. Topotecan abolished the increase (* $p=0.93$, ** $p=0.0004$, *** $p=0.056$, **** $p=0.0067$). (B) GLUT3 is increased in CHLA-20 neuroblastoma following bevacizumab and sunitinib treatment. Addition of low dose topotecan prevented this up-regulation, with levels similar to control (* $p=0.069$, ** $p=0.001$).

Fig. 4.

Addition of low dose topotecan decreased the number of CD34+ cells compared to anti-angiogenic therapy alone. (A) CD34+ cells were decreased with bevacizumab and sunitinib treatment but not topotecan. Treatment with the topotecan/bevacizumab combination decreased the total number of endothelial cells within tumor samples, suggesting a more potent anti-angiogenic effect, compared to monotherapy alone ($p=0.004$, $**p=0.378$). (B) Typical immunohistochemical staining showing areas of CD34+ cells (brown) in bevacizumab treated or bevacizumab/topotecan combination.