Effectiveness of the Angiogenesis Inhibitor TNP-470 in Reducing the Growth of Human Neuroblastoma in Nude Mice Inversely Correlates with Tumor Burden

Howard M. Katzenstein, Alfred W. Rademaker, Christof Senger, Helen R. Salwen, Nadine N. Nguyen, Paul S. Thorner, Louis Litsas, and Susan L. Cohn

Departments of Pediatrics [H. M. K., S. L. C.] and Pathology [C. S.], Children’s Memorial Hospital, Chicago, Illinois 60614; Robert H. Lurie Comprehensive Cancer Center, Northwestern University Medical School, Chicago, Illinois 60611 [H. M. K., S. L. C., A. W. R., H. R. S., N. N. N.]; and Department of Pediatric Laboratory Medicine, Division of Pathology, Hospital for Sick Children and University of Toronto, Toronto, MSG 1X8 Canada [P. S. T., L. L.]

ABSTRACT
Angiogenesis plays an important role in the growth and metastasis of malignant tumors. We have previously reported that in children with neuroblastoma (NB), tumor vascularity directly correlates with metastatic disease, MYCN amplification, and poor outcome. The angiogenesis inhibitor TNP-470 has been shown to reduce the rate of NB growth in rodents with macroscopic tumors without ultimately impacting survival. To investigate whether TNP-470 could more effectively inhibit NB growth in animals with a low tumor burden, we treated 30 nude mice with minimal disease with this angiogenesis inhibitor (supplied by TAP Pharmaceuticals, Inc.). Therapy was initiated before tumors were clinically evident after s.c. inoculation of \(5 \times 10^6\) cells from the MYCN-amplified NB cell line NBL-W-N. TNP-470 was administered 3 days/week, and after 12 weeks of treatment, 53% of the treated mice remained tumor free, whereas 100% of the control mice developed tumors \((P < 0.0001)\). To further assess the relationship between the efficacy of TNP-470 treatment and tumor burden, TNP-470 was also administered s.c., 3 days/week, to mice with clinically evident small (<400 mm\(^3\); \(n = 15\)) and large (>400 mm\(^3\); \(n = 11\)) tumors. For animals with small tumors, the mean rate of growth was significantly decreased in the treated mice compared to the controls \((P = 0.02)\). In contrast, there was no difference in the mean rate of tumor growth between animals with large tumors treated with TNP-470 and controls \((P = 0.64)\). Our studies demonstrate that the effectiveness of TNP-470 inversely correlates with tumor burden. We speculate that TNP-470 may most effectively inhibit NB tumor growth in children with a low tumor burden.

INTRODUCTION
Sustained growth and metastases of solid tumors are dependent on the development of an adequate blood supply through angiogenesis (1, 2). This process is mediated by an imbalance of angiogenic inducers and inhibitors released from both neoplastic cells and host cells (1–3). Numerous studies have demonstrated that outcome in adults with a variety of malignancies can be predicted by the degree of tumor angiogenesis (4–8). We previously reported that high tumor vascularity was associated with MYCN amplification, advanced-stage disease, and poor outcome in children with NB (9). Conversely, excellent survival was observed in the subset of children included in our analysis who had tumors with low vascular density. These observations suggest that high levels of angiogenic activators are likely to be present in NB tumors that are MYCN-amplified and rapidly progressive and that agents capable of interfering with the process of angiogenesis may inhibit or slow NB tumor growth. Thus, angiogenesis inhibitors may prove to be an effective modality of therapy for children with highly vascular, clinically aggressive NB tumors.

TNP-470, \((N\)-chloroacetyl-carbamoyl)-fumagillol, a semisynthetic analogue of fumagillin, is a potent inhibitor of angiogenesis (10). The antitumor effect of TNP-470 has been analyzed in a variety of experimental animal models, (11–14), and TNP-470 has recently been evaluated in Phase I and Phase II clinical trials (14, 15). In preclinical NB studies, TNP-470 treatment reduced the rate of primary tumor growth in tumor-bearing mice compared to control treatment. In addition, a decreased incidence of metastasis to the axillary lymph nodes and the liver was observed with TNP-470 treatment in one of the murine NB models. However, tumors continued to grow in the animals, and all mice eventually died from tumor progression.

To investigate whether TNP-470 would be more effective in the setting of minimal disease, we initiated treatment in nude mice before tumors were clinically apparent, either 12 h or 1

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2 To whom requests for reprints should be addressed, at Children’s Memorial Hospital, Division of Hematology/Oncology, Box 30, 2300 Children’s Plaza, Chicago, IL 60614. Phone: (773) 880-4562; Fax: (773) 880-3223; E-mail: scohn@nwu.edu.

3 The abbreviations used are: NB, neuroblastoma; MKI, mitotic-karyorrhexis index.
week after the mice were s.c. inoculated with cells from the human NB cell line NBL-W-N (16). We also treated animals 3–9 weeks after tumor cell inoculation, once small (<400 mm³) or large (>400 mm³) tumors developed, to evaluate whether the effect of TNP-470 treatment differed according to tumor burden. Our studies demonstrate that TNP-470 treatment does effectively inhibit NB growth when the agent is administered in the setting of minimal disease. Furthermore, when TNP-470 is administered to animals with small tumors, the rate of growth is reduced. However, TNP-470 treatment does not significantly alter the tumor growth rate when it is administered to animals with large tumors.

MATERIALS AND METHODS

Cell Culture. The MYCN-amplified human NB cell line NBL-W-N, a subclone of the NBL-W cell line, was established in our laboratory and has been described previously (16). The cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum plus antibiotics and glutamine (Life Technologies, Inc., Gaithersburg, MD).

TNP-470. TNP-470, a generous gift of TAP Pharmaceuticals, Inc. (Deerfield, IL), was stored at 4°C. A 10% stock solution of TNP-470 in ethanol was made weekly. For the animal studies, the stock solution was diluted 1:70 or 1:35 with PBS immediately before injection (Life Technologies, Inc.).

Animal Studies. Male 4–6-week old homozygous athymic nude mice were obtained from Harlan Sprague Dawley (Madison, WI). The mice were housed in a laminar flow caging system, fed ad libitum, and treated in accordance with a protocol approved by the Northwestern University Animal Care and Use Committee. All mice were inoculated s.c. with 5 × 10⁶ NBL-W-N cells in the right flank.

A total of 30 mice with minimal disease received TNP-470 (30 mg/kg) s.c. at a distant site in three different experiments, beginning either 12 h (n = 21) or 1 week (n = 9) after tumor cell inoculation, before tumors were clinically evident. Subsequently, the mice were treated with TNP-470 (7.5 mg/kg) twice daily on Monday, Wednesday, and Friday for 12 weeks. To investigate whether prolonged administration of TNP-470 would enhance survival, a subgroup of four mice received prolonged treatment with TNP-470 for a total of 17 weeks. Mice were observed for a total of 12 months after tumor cell inoculation and then euthanized. Control mice (n = 16) received injections at the same intervals with vehicle alone (1% ethanol in PBS).

In separate studies of mice with macroscopic disease, TNP-470 (30 mg/kg) was administered s.c. once daily on Monday, Wednesday, and Friday to mice (n = 15) with clinically evident small tumors (defined as <400 mm³) and mice (n = 11) with large tumors (>400 mm³). At the same time, 13 control mice with large (n = 5) or small (n = 8) tumors were treated with vehicle alone. Tumor volume was measured weekly using the formula tumor volume = (length × width²)/2 (17). Mice were sacrificed at the first sign of illness or when they had clinical evidence of discomfort from the tumor.

Proliferation and Cytotoxicity Assays. NBL-W-N cells were seeded into 96-well tissue culture plates at a concentration of 5 × 10⁴ cells/well in complete DMEM. After the cells were incubated for 24 h at 37°C, test media containing TNP-470 in concentrations ranging from 1 mg/ml (2.5 mM) to 1 µg/ml (2.5 µM) or control media containing 1% ethanol were added to the wells. The cells were refed 48 h later with fresh test media and incubated at 37°C for an additional 24 h, and the number of proliferating cells was then measured using the CellTiter 96 A Q nonradioactive cell proliferation assay (Promega Corp., Madison, WI). The number of proliferating cells in the cultures containing TNP-470 was compared to the number of cells cultured in control media (100%). All conditions were assayed in quadruplicate. Similar conditions were used to assess the cytotoxic effects of TNP-470 on the NBL-W-N cells. The number of surviving cells cultured in media containing TNP-470 in concentrations ranging from 1 mg/ml (2.5 mM) to 1 µg/ml (2.5 µM) was determined using trypan blue exclusion and compared to the number of surviving cells cultured in the control media.

Histopathology and Immunohistochemistry. Tumor tissue for histological examination was obtained from moribund animals immediately after they were sacrificed. In addition, tumor tissue was processed for histology in a small number of control and treated mice sacrificed before they exhibited signs of illness, after 2 weeks of treatment. Tumor tissue was fixed in 10% buffered formalin and embedded in paraffin. Four-µm-thick histological sections were cut for H&E staining. Histological analyses were performed without knowledge of animal treatment. The MKI was calculated for each case according to the criteria described by Shimada et al. (18). The percentage of necrosis was estimated after examining at least ten ×400 fields.

Determination of Microvessel Density. To highlight endothelial cells, immunohistochemical staining with mouse monoclonal anti-CD31 antibody (Cell Marque, Austin, TX) was performed on additional formalin-fixed and paraffin-embedded tumor sections using an automated stainer (Ventana, NEXES, Tucson, AZ) according to the manufacturer’s instructions. Microvessel number was assessed in tumor areas showing the highest density of staining as determined by an initial scan with low magnification (×20), as described previously (5). For vessel counting, a ×200 field in each of three to four of the most vascular areas was counted, and the average counts were recorded. Structures clearly identifiable as vessel or vascular buds (vessels without a clear vascular lumen) were included in the count. Vessels with a diameter of >50 µm were excluded. Vessels in fibrovascular septa were counted only once if they were identified as the same vessel. Vessel counting was performed without knowledge of the treatment the animal received.

Apoptosis Analysis. Determination of apoptosis in situ was performed based on the terminal deoxynucleotidyl transferase-mediated nick end labeling assay using the ApopTag Kit (Intergen Company, Purchase, NY) according to the manufacturer’s directions. Stained slides were examined on a Leitz Dialux (Leica Canada) photomicroscope with a color video camera (Pulnix TMC-7; Bock Optronic, Inc.) connected to it. The video camera was attached to a personal computer Pentium II (Window’s NT 95). A video image of the slide showing positive and negative nuclei was created on the computer monitor using the ATI Version 4.35 video imaging program using a ×10 ocular and a ×25 objective. The video image was captured and analyzed using Scion Image 1998 software (Scion Corp.). Positive and negative nuclei were scored separately. A total of
RESULTS

In Vivo Tumor Growth. To investigate whether TNP-470 would effectively inhibit NB growth in animals with minimal tumor burden, 46 mice were inoculated with NB cells and treated with either TNP-470 or control vehicle before the emergence of clinically palpable tumors. After 12 weeks of treatment, 16 of 30 (53%) mice treated with TNP-470 were tumor free, and 23 (77%) mice were alive. Over a median follow-up of 11 months (range, 4–12 months), 14 of the TNP-470-treated mice remained disease free, 13 mice died from local tumor progression, and 3 mice died secondary to toxicity, including two mice from the subgroup that received prolonged TNP-470 therapy. There was no statistical difference in tumor-free survival between the animals treated 12 h after tumor inoculation and those initially treated at 1 week (P = 0.75). In contrast, within the same 12-week period of time, tumors developed in all 16 (100%) control mice, and 15 (94%) animals died from tumor progression (P < 0.001; Fig. 1).

To further investigate the relationship between tumor burden and the effectiveness of TNP-470 treatment in retarding NB growth, treatment was also administered to mice with clinically apparent small (<400 mm³) or large (>400 mm³) tumors. A significant decrease in mean tumor growth rate was observed when TNP-470 was administered to mice bearing small tumors compared to that seen in the control animals (36 ± 8 mm³/day versus 98 ± 24 mm³/day, respectively; P = 0.02; Table 1). In addition, after 3 weeks of treatment with TNP-470, the average tumor volume in mice with small tumors was significantly lower than that observed in controls (P = 0.02; Fig. 2A). In contrast, the mean rate of tumor growth in the animals with large tumors treated with TNP-470 did not differ significantly from that seen in the control animals bearing large tumors (259 ± 47 mm³/day versus 220 ± 69 mm³/day respectively; P = 0.64). Similarly, after 2 weeks of therapy, the average tumor volume in TNP-470-treated mice with large tumors did not differ significantly from that of controls (P = 0.93; Fig. 2B). In this model, none of the animals developed metastatic disease.

Histology. The xenografts from both the treated and control animals were composed of sheets of neuroblasts with scant cytoplasm (Fig. 3). There was almost no connective tissue and no evidence of Schwannian stroma or neuropil. A small number of neuroblasts exhibited some evidence of morphological differentiation with increased amounts of cytoplasm and vesicular nuclei. However, complete ganglionic differentiation was not observed. The mean MKI was similar in each of the subgroups analyzed (Table 1). There was no significant difference in the number of apoptotic cells or in the percentage of necrosis detected in the tumors from the animals treated with TNP-470 versus control vehicle. Similarly, the number of vessels counted in the treated tumors was not significantly different from that observed in the control tumors.

Toxicity. Treatment was generally well tolerated in the animals treated with TNP-470 at a dose of 30 mg/kg/day, three times/week. During the 12-week treatment regimen, 4 of 30 mice developed cachexia and weight loss, which resolved when the drug was discontinued. To discern whether continued therapy would result in a further improvement in survival, a subgroup of four mice continued to receive treatment for a total of 17 weeks. However, therapy was discontinued when similar but irreversible toxicity occurred in two of the four mice. In addition, one of the treated mice developed a palpable tumor 8 weeks after tumor inoculation and died days later of a pulmonary hemorrhage.

In Vitro Proliferation and Cytotoxicity Studies. In vitro proliferation and cytotoxicity assays were performed to determine whether the antitumor effect of TNP-470 was angio-static, cytostatic, and/or cytotoxic. NBL-W-N cell proliferation was not affected by TNP-470 in concentrations ranging from 1 pg/ml to 1 μg/ml. However, in the presence of 10 μg/ml (25 μM) TNP-470, NBL-W-N cell proliferation was inhibited by 16% compared to control. Proliferation was completely blocked at TNP-470 concentrations of 100 μg/ml (250 μM; Fig. 4). Cytotoxicity of the NBL-W-N cells was first observed at TNP-470 concentrations of 100 μg/ml (250 μM; data not shown).

DISCUSSION

This study demonstrates that the effectiveness of the angiogenesis inhibitor TNP-470 is inversely related to NB tumor
Angiogenesis Inhibition in Neuroblastoma

Table 1 Effect of TNP-470 on the growth rate, histopathology, and vascularity of NB xenografts

<table>
<thead>
<tr>
<th></th>
<th>Small tumors (&lt;400 mm³)</th>
<th>Large tumors (&gt;400 mm³)</th>
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<tr>
<td></td>
<td>Controls (Mean ± SE (n))</td>
<td>TNP-470-treated mice (Mean ± SE (n))</td>
</tr>
<tr>
<td>Rate of tumor growth (mm³/day)</td>
<td>98 ± 24 (8)</td>
<td>36 ± 8 (15)</td>
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<tr>
<td>MKI/5000 cells</td>
<td>154 ± 9 (7)</td>
<td>158 ± 21 (12)</td>
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<tr>
<td>No. of blood vessels/high-power field</td>
<td>61 ± 6 (7)</td>
<td>67 ± 9 (9)</td>
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<tr>
<td>Tumor necrosis (%)</td>
<td>12 ± 6 (7)</td>
<td>12 ± 4 (12)</td>
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a NS, not significant.

Fig. 2 A, tumor growth of NB xenografts in animals bearing small tumors (<400 mm³) treated with TNP-470 (n = 15) versus control vehicle (n = 8). The mean ± SE of tumor volume is shown. B, tumor growth of NB xenografts in mice bearing large tumors (>400 mm³) treated with TNP-470 (n = 11) versus control vehicle (n = 5). The mean ± SE of tumor volume is shown.

burden. TNP-470 was administered to three groups of mice after s.c. inoculation with cells from the MYCN-amplified human NB cell line NBL-W-N (16). NBL-W-N cells were used in our studies because previous experiments had demonstrated that virtually 100% of animals inoculated with these cells develop tumors (20). The first group of mice had minimal disease and was treated shortly after inoculation with NB cells, before tumors were clinically evident; the second group received treatment once they developed small (<400 mm³) tumors; and the third group was treated after their tumors were >400 mm³. After 12 weeks of treatment with TNP-470, 53% of the animals with minimal disease were tumor free. Furthermore, 14 of these animals have remained tumor-free after being followed for a median of 11 months. In contrast, tumors developed in all of the control mice 3–9 weeks after tumor inoculation. In animals bearing small tumors, TNP-470 treatment decreased the mean rate of tumor growth, although tumors continued to increase in size over time. However, when TNP-470 was administered to animals with large tumors, the mean tumor growth rate did not differ significantly from that observed in control animals. Thus, TNP-470 inhibited tumor growth in animals with minimal disease and decreased the rate of growth in animals bearing small tumors. This model suggests that there may be a critical NB tumor burden above which TNP-470 has little efficacy.

Similar to other series, a small number of animals treated with TNP-470 in our study developed weight loss (12, 21). Although three animals died from complications related to TNP-470 treatment, overall survival for the group of animals whose treatment was initiated when disease was minimal remained superior to that of the control group. The TNP-470 dose we used in our studies was similar to the regimens described by other investigators in preclinical trials (11, 12, 14). Limited data exist on the pharmacokinetics of TNP-470 and its known metabolites, and t½ values range from 7 min to 3 h (14). This prompted our use of a smaller dose given twice daily on treatment days for the mice with minimal disease. Additional experiments are planned to evaluate whether treatment with a further reduction in dose will result in decreased toxicity but similar response rates.

Although TNP-470 is a known inhibitor of endothelial cell proliferation (22), the mechanisms by which TNP-470 inhibits tumor growth remain largely unknown. To determine whether TNP-470 affected NBL-W-N cell growth directly, we performed tumor cell proliferation and cytotoxicity assays. Inhibition of NBL-W-N cell proliferation was not observed until the concentration of TNP-470 was ≥10 μg/ml, and cytotoxic effects were not seen until the concentration of TNP-470 was ≥100 μg/ml. Because the plasma concentrations of TNP-470 in animals after s.c. injections have been reported to be <1 μg/ml (12), the in vivo antitumor effect observed in our studies does not appear to be due to decreased NB tumor cell proliferation or a direct cytotoxic effect.

Histological analyses were performed to compare the tumors from the treated and control groups, and no obvious
morphological differences were observed. Our inability to detect differences in the degree of apoptosis among the different treatment groups may be explained in part by the high level of apoptosis that occurs in untreated NB tumors (23, 24). Furthermore, necrosis frequently occurs in large tumors that outgrow their vascular supply, and the majority of the tumors examined histologically were >1000 mm$^3$. Surprisingly, we also found that blood vessel density was not affected by TNP-470 treatment, indicating that some blood vessels can remain intact after treatment with this agent. Interestingly, Bergers et al. (25) also reported little change in blood vessel density with angiogenesis inhibitor treatment in a transgenic model of pancreatic islet cell carcinogenesis. These authors speculated that the intact vessels somehow dictate the number of tumor cells that can survive, thus governing tumor size while maintaining a similar density of tumor vessels.

The results of this study and the preclinical trials reported by others (10–12, 26) demonstrate that the rate of tumor growth is decreased with TNP-470 treatment. However, TNP-470 appears to have only limited antitumor activity in animals with clinically evident tumors because tumors do not regress with TNP-470 therapy, and long-term survival is not affected by treatment. In contrast, survival was significantly improved in the group of animals that received TNP-470 treatment 12 h or 1 week after tumor inoculation, indicating that this agent may be most effective in inhibiting NB growth in the setting of minimal disease. Our results further suggest that little clinical response may be observed in Phase I and II studies of TNP-470, in which most patients have large, bulky tumors. However, TNP-470 may prove to be effective in children with NB if it is administered after the completion of intensive multimodality therapy, when only minimal residual disease is present. In an effort to further test this hypothesis and to more closely mimic the clinical scenario of minimal disease, additional studies are ongoing in which animals with palpable tumors are being treated with chemotherapy followed sequentially by TNP-470.

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REFERENCES

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