Abstract. Background: High risk neuroblastoma (NB) patients have an overall five-year survival of ~50%, indicating the need for new treatment strategies, such as angiogenesis inhibition. Materials and Methods: The angiogenesis inhibitor TNP-470 (30 mg/kg, every other day, subcutaneously) was given to nude mice with subcutaneous human neuroblastoma xenografts. The plasma concentrations of the angiogenesis stimulators, i.e. vascular endothelial growth factor A (VEGF-A), fibroblast growth factor 2 (FGF-2) and hepatocyte growth factor (HGF), were assayed longitudinally. Angiogenesis, proliferation and apoptosis were quantified on tumor tissue slides. Results: Upon treatment with TNP-470, angiogenesis was significantly inhibited by the reduction of length and surface area of vessels per tumor volume, without having significant effect on tumor growth, tumor cell proliferation or apoptosis. Plasma concentrations of VEGF-A per tumor volume were significantly increased upon treatment. Conclusion: Angiogenesis inhibition must reach a threshold before significant tumor cell apoptosis and a reduction of the tumor growth rate occur.

Inhibition of angiogenesis as a strategy to suppress tumor growth was proposed by Folkman in the early 1970s (1). Now, three decades later, more than 30 angiogenesis inhibitors have entered clinical trials and three of them have shown clinical efficacy. TNP-470 was one of the first identified exogenous angiogenesis inhibitors and is now tested in Phase II clinical trials (2). TNP-470 is a synthetic analog of the fungal antibiotic fumagillin and inhibits endothelial cell proliferation and migration (3-6).

Neuroblastoma (NB) is a rapidly growing, highly vascularized tumor mostly affecting young children. The prognosis for disseminated NB is still poor (7). It has been shown that vascular endothelial growth factor A (VEGF-A) is expressed in NB and is involved in tumor progression (8-10). Additionally, it has been found that patients with NB frequently exhibited elevated serum concentrations of VEGF-A and hepatocyte growth factor (HGF), and that high concentrations of these pro-angiogenic factors are correlated to advanced stages and a poor outcome (Sköldenberg et al., in preparation). TNP-470 inhibits the growth of NB xenografts in rats and mice (11-14). In the present investigation the effects of the angiogenesis inhibitor TNP-470 on NB xenografts in nude mice was studied in relation to plasma concentrations of VEGF-A, fibroblast growth factor 2 (FGF-2) and HGF.

Materials and Methods

Cell cultures. SH-SY5Y cells derived from a poorly differentiated human NB tumor (15) were maintained as previously described (14). Bovine adrenal capillary endothelial cells (BCE) were harvested from calf adrenal gland cortex as described by Qi et al. (16). The BCE cells were grown in DMEM with L-alanyl-L-glutamine, sodium pyruvate, 4,500 mg/l D-glucose and pyridoxine supplemented with 10% newborn calf serum, penicillin and streptomycin and 3 ng/ml FGF-2 (Sigma Chemical Co., St. Louis, MO, USA).

Cytotoxicity assay. The cells were plated on flat-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) at a density of 20,000 cells/well (0.33 cm²) for the SH-SY5Y cells, and 5,000 to 10,000 cells/well for the BCE cells. The cells were plated 24 h prior to addition of the drugs. Various TNP-470 concentrations (Takeda Chemical Industries, Ltd.; Osaka, Japan) ranging from 2.5 nM to 25 µM were added to the wells. The TNP-470 was first dissolved 1:10 (w/v) in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany), and then in distilled water to the desired concentrations. On each microtiter plate, six wells with cells and medium, and three wells with cells, medium and vehicle without drugs served as controls. Six wells were used as blank wells with medium alone.

The survival index of the cells in each well was measured 72 h after addition of the drug by a fluorometric microculture
second day were randomized to receive either TNP-470 (30 mg/kg injected every second day until the tumor reached a volume of 0.3 ml, the treatment began. The animals were given water and food pellets. All handling of the animals was carried out under aseptic conditions. The animals' weight and general appearance were recorded every other day throughout the experiment. The regional ethics committee for animal research approved the experiment. The inoculation of tumor cells was performed subcutaneously (s.c.) in the right hind leg, as previously described (14).

Animals and tumor transplantation. Male NMRI nu/nu mice (B&M, Ry, Denmark) were used for xenografting at an age of 6 weeks (body weight 25-30 g). The animals were housed at a temperature of 24°C, with a 12-h light, 12-h dark cycle. They were fed ad libitum with water and food pellets. All handling of the animals was carried out under aseptic conditions. The animals' weight and general appearance were recorded every other day throughout the experiment. The regional ethics committee for animal research approved the experiment. The inoculation of tumor cells was performed subcutaneously (s.c.) in the right hind leg, as previously described (14).

Measurement of tumor volume and administration of drugs. The TNP-470 was suspended in 1% ethanol and 5% gum arabic in 0.9 mg/ml saline solution. Tumor volume measurement began when the tumor became palpable (~0.1 ml) and was then repeated every second day. Each animal was anesthetized, and the longest diameter of the tumor and the width perpendicular to it were measured with a caliper. The tumor volume was then calculated as: length x width^2 x 0.44. When a tumor reached a volume of 0.3 ml, the treatment began. The animals were randomized to receive either TNP-470 (30 mg/kg injected every second day s.c. in the neck, n=10) or vehicle control (n=11). This dose of TNP-470 has been shown to be efficient in multiple xenograft mouse tumor models (18) but the effect of this dose on treatment of NB is conflicting. Higher doses have however caused weight loss (19).

Perfusion fixation and autopsy. At termination of the experiment, the animals were anesthetized with an i.p. injection of 25 mg/kg of 2,2,2 tribromoethanol (Sigma) in 2.5% 2-methyl-2-butanol (Sigma) in 0.9 mg/ml saline solution. A cannula was inserted in the thoracic aorta, and the animal was whole body perfusion-fixed at 100 mm Hg with 4% paraformaldehyde in 1.47 mg/ml NaH2PO4 x H2O, 12.62 mg/ml Na2HPO4 x 2 H2O and 4.09 mg/ml NaCl in distilled H2O (Millonig's buffer, pH 7.4, 37°C).

Tissue analyses. The paraformaldehyde-fixed tumors were dehydrated and embedded in paraffin. Tissue sections 3-μm thick were cut and put on diaminoalkyl-silane-treated glass slides, dehydrated and stained for apoptotic cells by TUNEL and for proliferating cells by Ki-67 nuclear antigen immunohistochemistry, as previously described (20).

Stereological quantifications. Stereological estimates of vascular parameters were performed on hematoxylin and eosin stained tissue sections. One observer quantified all sections in a blinded manner. Structures were counted at x400 with an eyepiece grid (506800, Fluroscope, Illnau, Switzerland) of 10x10 squares (0.25x0.25 mm). The grid was placed at random at the upper left corner of a section, and then systematically advanced every 1 to 3 mm (depending on the tumor size) in both directions with use of the microscope’s goniometer stage. Morphological parameters from 20-26 grids were quantified for each tumor. Areas with hemorrhage and apoptotic or necrotic cells were considered as non-viable and were excluded from analysis of other parameters (20), but were used for calculation of the viable tissue fraction. The blood vessels were visible as punched-out holes in stained sections after perfusion fixation, and hence no specific endothelial cell marker was necessary. The length of vessels per tumor volume (Lv) and the surface area of vessels per tumor volume (Sv) were calculated as shown in Table 1 (20).

The mean radius of viable tumor tissue supported by one blood vessel was determined. Half the distance between two blood vessels, or the distance from one blood vessel to a non-viable area, was recorded (Image-Pro Plus 3.0, Media Cybernetics, Silver Spring, MD, USA). Three such radii were recorded from each grid (see below), and a total of 20-26 grids were quantified per tumor.

The fractions of Ki-67-positive, as a sign of proliferating cells, and TUNEL-positive tumor cells, indicating apoptosis, were determined among 2,000 cells in each section.
Plasma levels of angiogenesis stimulators. During treatment, blood samples were taken from the retro-orbital venous plexus once a week with heparinized capillary tubes (Drummond Scientific Company, Broomall, PA, USA). Prior to perfusion, sampling of blood was performed from the left atrium with heparinized syringes. The collected blood was spun within 30 min at 1,000 xg for 20 min. The plasma was removed and stored at –20°C, pending analysis for the angiogenic peptides VEGF-A, FGF-2 and HGF. For these measurements, specific quantitative colorimetric sandwich ELISAs (R&D Systems, Minneapolis, MN, USA) were made according to the manufacturer’s instructions.

Statistical analysis. Data were processed in Statistica 5.0 (StatSoft Inc., Tulsa, OK, USA). Differences between groups were analyzed with the Mann-Whitney U-test and the level of significance was set at \( p<0.05 \).

Results

In vitro toxicity of TNP-470. At concentrations below 2.5 mM, TNP-470 decreased the BCE cell viability by 30%, but did not affect SH-SY5Y cell viability (Figure 1). At higher concentrations, the TNP-470 was more toxic to the SH-SY5Y cells than to the BCE cells (Figure 1).

TNP-470 reduced angiogenesis without reducing tumor growth or viability in vivo. The TNP-470 was administered for 18 days, when the experiment was terminated because of tumor progression. The TNP-470 slowed tumor growth by 31% (mean volume of the treated tumors divided by the mean volume of control tumors (T/C) was 0.69 at day 18 of treatment) without reaching significance \( (p=0.20, \text{ Figure 2}); \) No metastases were seen.

Although tumor cell apoptosis was increased and proliferation was decreased in TNP-470 treated animals, the differences were not statistically significant when compared to controls (Table II).

Quantification of tumor vascular parameters showed significant inhibition of angiogenesis by a 38% decrease in \( S_v \) and 42% decrease in \( L_v \) (Figure 3 and Table III). A non-significant decrease in the mean viable tissue fraction (vc) of the tumors was observed in the TNP-470 treated animals compared to controls.

Plasma VEGF concentrations increased during treatment. VEGF-A plasma concentrations were increased, but the difference was not significant. When adjustment was made

<table>
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<tr>
<th>Table II. Quantification of tumor dynamics at day 18. Mean ± 1 S.D.</th>
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<td>Control (n=9/10)</td>
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<tr>
<td>Viable fraction (%)</td>
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<tr>
<td>Proliferation (%)</td>
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<tr>
<td>Apoptotic cells (%)</td>
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Differences between groups were not significant (Mann-Whitney U-test).
for differences in tumor volume, the VEGF increase corresponded to a 198% increase per tumor volume compared to untreated controls \((p<0.05)\). The FGF-2 concentrations in TNP-470-treated animals and control animals were similar at days 7 and 14 of treatment and fell on day 18 (Table IV). The plasma HGF concentrations in all samples were below the detection limit of the assay.

**Discussion**

It is known that TNP-470 irreversibly inactivates the enzyme methionine aminopeptidase2 (MetAP2) (21). Further, TNP-470 is an angiogenesis inhibitor that blocks endothelial cell (EC) proliferation and migration (6, 14, 22, 23), the exact mechanism of which is not known (4).

Recently it has been shown that TNP-470 also inhibits increased vascular permeability and EC migration in response to VEGF \textit{in vivo}, and requires both a leaky environment and proliferating EC to be effective (19). TNP-470 also decreases

<table>
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<th>Table III. Quantification of tumor angiogenesis. Mean ± 1 S.D.</th>
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<tr>
<td>Control ( (n=9/10) )</td>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Lv (mm(^{-2}))</td>
</tr>
<tr>
<td>Vv ( (10^{-3}) )</td>
</tr>
<tr>
<td>Sv (mm(^{-1}))</td>
</tr>
<tr>
<td>d ((\mu m))</td>
</tr>
<tr>
<td>R ((\mu m))</td>
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Mann-Whitney \( U \)-test where \( *p<0.05 \); Lv, length of vessels per tumor volume; Vv, Volume of vessels per tumor volume; Sv, surface area of vessels per tumor volume; d, mean section diameter of vessels; R, radius of tumor tissue supported by one blood vessel = mean distance between two adjacent vessels.

VEGFR-2 phosphorylation, intracellular Ca\(^{2+}\) concentration and RhoA activation in EC which are necessary for EC proliferation and migration (24). In the present study TNP-470

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Figure 3. Representative neuroblastoma tumor sections stained with eosin \( (a, b; x10) \) or Bandeiraea simplifolia-I lectin, staining of endothelial cells \( (c, d; x20) \). All sections were counterstained with hematoxylin. Note areas of necrosis in \( a \) and \( b \) (arrows). \( a \) and \( c \): controls; \( b \) and \( d \): TNP-470 treatment; bar = 50 \( \mu m \).
activity, on the other hand, has resulted in increased dose-escalation. Escalating the dose in order to achieve better antitumor effects if such an inhibitor was to be combined with other therapies (28-30).

Acute and subacute systemic toxicity studies support the concept of MetAP2 inhibition, the target of TNP-470, as a therapeutic approach in cancer and other angiogenesis dependent diseases (21).

In a previous study (14) TNP-470 (10 mg/kg) gave a 64% reduction in NB tumor growth in nude rats while in the present nude mouse study (30 mg/kg), the effect was not significant. Treatment began at a tumor volume of 0.3 ml in both species (approximately 1% of BW in mice, 0.2% in rats). This difference in efficacy may not only be explained by the differences in species and in immunological properties, but also in tumor relative size. The literature is conflicting regarding the role of tumor size on the efficacy of angiogenesis inhibitors, some showing a better effect in large (38) and others in small tumors (39, 40). Interestingly, TNP-470 does not prevent angiogenesis or the transition from microscopic to macroscopic metastases despite reducing the total tumor burden (41). Therefore, the potency of an angiogenesis inhibitor in animal models may depend on the tumor type, the tumor burden, the tumor growth rate, the host species, the dose, dosing interval and the route of administration of the agent.

Table IV. Circulating plasma concentrations of human VEGF-A and FGF-2. Mean ± 1 S.D.

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<th></th>
<th>7 Days</th>
<th>14 Days</th>
<th>18 Days</th>
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<tr>
<td></td>
<td>Control</td>
<td>TNP-470</td>
<td>Control</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>125±9.17</td>
<td>125±6.76</td>
<td>160±87.1</td>
</tr>
<tr>
<td>VEGF/ tumor vol.</td>
<td>144±62.1</td>
<td>182±153</td>
<td>119±40</td>
</tr>
<tr>
<td>FGF-2</td>
<td>395±222</td>
<td>393±186</td>
<td>316±165</td>
</tr>
<tr>
<td>FGF-2/ tumor vol.</td>
<td>491±443</td>
<td>628±791</td>
<td>178±81</td>
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Concentrations in pg/ml. Mann-Whitney U-test where *p*<0.05.

reduced the survival of both BCE and SH-SY5Y cells in vitro; however, a threshold of >2.5 μM had to be reached before inhibition of EC proliferation occurred.

Such a threshold phenomenon was also demonstrated in vivo, since TNP-470 significantly reduced blood Lv and Sv without having significant effects on net tumor growth, tumor cell proliferation or apoptosis. A threshold hypothesis is also supported by our observation that the mean radius of tumor tissue supplied by one vessel was similar in treated and untreated animals, since a continuous effect was achieved by more frequent administration (25) or by continuous infusion (12) of TNP-470, is likely to induce an increase in vascular distance. The presence of a threshold for angiogenesis inhibition in vivo can be explained by the observation that the tumors are comparatively overvascularized and that angiogenesis was not reduced to a sufficient extent for cell death to occur (26). Additionally, a threshold for angiogenesis inhibition indicates the possibility that the “failure” of some of the angiogenesis inhibitors in early clinical trials was due to unsuitable surrogate markers, dosing, or combination therapies.

The increase seen in plasma VEGF-A per tumor volume suggested that TNP-470 may have enhanced hypoxia by angiogenesis inhibition despite unaltered tumor cell viability. The tumor cells might have up-regulated VEGF not only as an angiogenesis stimulator, but also as an autocrine survival factor to reduce the effects of metabolic stress (9, 27). This could be more applicable to the SH-SY5Y cells chosen for this study since such cells have the ability to produce high levels of VEGF and even express receptors for this factor. The lack of significant reduction of tumor growth by an angiogenesis inhibitor in our study does not exclude the possibility of additive or synergistic effects if such an inhibitor was to be combined with other therapies (28-30).

An angiogenesis inhibition threshold could perhaps be surpassed by more frequent administration (25) or by continuous infusion (12) since TNP-470 and its active metabolite have a short plasma half-life in humans (31). Escalating the dose in order to achieve better antitumor activity, on the other hand, has resulted in increased dose-limiting neurotoxicity (31, 32). Combination therapy of TNP-470 with different cytotoxic drugs has shown synergistic effects (33). Recently, it has been demonstrated that conjugation of TNP-470 to an N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer increased delivery of TNP-470 to tumor vessels because of the enhanced permeability and retention (EPR) effect. The EPR effect in tumors depends on the enhanced permeability and poor lymphatic drainage of tumor vasculature leading to increased drug retention (8, 19, 34). It has been shown that the EPR effect increases with increasing molecular weight of the molecule entering the tumor (34, 35). The polymer conjugated TNP-470, however, must release the active TNP-470 (through the action of lysosomal cysteine proteases that are overexpressed in the tumor) for the molecule to be able to interact with MetAP2 in endothelial cells (19). Additionally, some new analogs of TNP-470 with better pharmacokinetics have been identified. Of these new analogs, the reversible MetAP2 inhibitor A-357300 has higher specific MetAP2 inhibitory activity, a longer plasma half-life, and potently reduces tumor growth in vivo (36, 37). These studies support the concept of MetAP2 inhibition, the target of TNP-470, as a therapeutic approach in cancer and other angiogenesis dependent diseases (21).
In summary, TNP-470 can reduce tumor angiogenesis without significant reduction of tumor growth. A threshold, a minimal inhibition of angiogenesis, may have to be reached before tumor cell apoptosis is triggered and a reduction in tumor growth rate occurs which may explain the modest clinical efficacy of some angiogenesis inhibitors as single agents. The existence of such an angiogenesis inhibition threshold must however be confirmed in other tumor models.

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References


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