High-Level Expression of Angiogenic Factors Is Associated with Advanced Tumor Stage in Human Neuroblastomas

Angelika Eggert, Naohiko Ikegaki, Janet Kwiatkowski, Huaqing Zhao, Garrett M. Brodeur, and Bruce P. Himelstein
Division of Oncology, The Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania 19104

ABSTRACT

Angiogenesis is essential for tumor growth and metastasis and depends on the production of angiogenic factors by tumor cells. Neuroblastoma (NB) is a common pediatric tumor of neural crest origin, which is biologically and clinically heterogeneous. Increased tumor vascular index correlates with poor outcome of NB. To determine which angiogenic factors contribute to NB angiogenesis and thereby support tumor progression, we examined the expression of eight angiogenic factors [vascular endothelial growth factor (VEGF), VEGF-B, VEGF-C, basic fibroblast growth factor, angiopoietin (Ang)-1, Ang-2, transforming growth factor α, and platelet-derived growth factor (PDGF)] by semiquantitative RT-PCR in 37 NB primary tumors and in 22 NB cell lines. We also analyzed the relationship between angiogenic factor expression and clinicopathological factors as well as patient survival. All eight angiogenic factors examined were expressed at various levels in NB cell lines and tumors, suggesting their involvement in NB angiogenesis. The expression levels of most angiogenic factors were correlated with each other, suggesting their synergy in regulating the angiogenic process. Significantly higher expression levels of VEGF, VEGF-B, VEGF-C, basic fibroblast growth factor, Ang-2, transforming growth factor α, and PDGF-A (P < 0.0001–0.026) were found in advanced-stage tumors (stages 3 and 4) compared with low-stage tumors (stages 1, 2, and 4S). Expression of PDGF-A was significantly associated with patient survival (P = 0.04). The redundancy in angiogenic factor expression suggests that inhibition of VEGF bioactivity alone might not be a sufficient approach for antiangiogenic therapy of human NB.

INTRODUCTION

Angiogenesis is essential for tumor growth and metastasis formation (1, 2). Numerous angiogenic factors that regulate this complex process alone or in synergy have been identified (3). VEGF, bFGF, TGF-α, PDGF, Ang-1, and Ang-2 have been shown to induce angiogenesis in a variety of experimental models (4–11).

VEGF, also known as vascular permeability factor, is an important angiogenic agent and endothelial-specific mitogen, which has been implicated in the neovascularization of a wide variety of tumors (12–16). VEGF acts via a paracrine mechanism mainly through two specific receptors on the surface of endothelial cells: Flt-1 and KDR (17, 18). Although encoded by a single gene, VEGF has several isoforms generated by alternative splicing (19, 20). Of these, the main isoforms, VEGF121 and VEGF165, are secreted soluble glycoproteins, whereas VEGF189 and VEGF206 remain bound to heparan sulfate proteoglycans at the cell surface (21). The importance of VEGF as a potential target for antineoplastic therapy has been demonstrated in several studies in which neutralizing antibodies to VEGF inhibited tumor growth and vascularization in vivo (22, 23).

VEGF-B and VEGF-C are two recently discovered members of the VEGF family (24–26), which are expressed in many tissues and have mitogenic and/or chemotactic actions on endothelial cells, indicating that they may also contribute to the induction or maintenance of angiogenesis. VEGF-C was discovered as the ligand for the third member of the VEGF receptor family (Flt-4, or VEGF receptor 3), which is expressed mainly on lymphatic endothelium of adult tissues (26, 27). VEGF-C is also angiogenic in vivo (11). Recently, expression of VEGF-B and VEGF-C has been detected in a variety of human tumors (28).

Ang-1, the ligand for TIE-2, a receptor-like tyrosine kinase expressed almost exclusively in endothelial cells, seems to be important to maintain vessel integrity by mediating interactions between the endothelium and surrounding matrix (10, 29). Because it stabilizes the structure of newly formed vessels, it has a later role in angiogenesis than VEGF. Its naturally occurring antagonist, Ang-2, binds with similar affinity to TIE-2, but does not activate the receptor (7). Inhibition of Ang-1 by Ang-2 has been suggested to drive angiogenesis in the presence of angiogenic inducers like VEGF by loosening contacts between endothelial and periendothelial cells, thus rendering endothelial cells accessible to angiogenic inducers (6).

bFGF is a mitogenic, angiogenic, and neurotrophic factor

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2 To whom requests for reprints should be addressed, at Children’s Hospital of Philadelphia, Division of Oncology, ARC Room 902, 3516 Civic Center Boulevard, Philadelphia, PA 19104. Phone: (215) 590-4855; Fax: (215) 590-3770; E-mail: eggert@email.chop.edu.
3 VEGF, vascular endothelial growth factor; bFGF, basic fibroblast factor; TGF-α, transforming growth factor α; PDGF, platelet-derived growth factor; Ang, angiopoietin; NB, neuroblastoma; RT, reverse transcription.
expressed by many tumor cells (30–33). PDGF consists of two related polypeptides (A- and B-chain) (34, 35). It originally was known to be involved in the regulation of cell migration and proliferation, but it has more recently been found to possess an angiogenic capability both in vitro and in vivo (8). TGF-α has been shown to induce VEGF expression (36) and has also an angiogenic role in vivo (37, 38).

NB is the most common extracranial malignant solid tumor of childhood and arises from the sympathetic nervous system. A number of different biological and genetic factors are known to influence the heterogeneous biological and clinical behavior of NBs (39). The invasive, metastatic, and hypervascular nature of high-stage NB may be one of the key obstacles to the cure of this disease. Evidence suggests that higher vascularity in NB correlates with metastasis, MYCN amplification, unfavorable histology, and poor outcome (40). Hence, it is likely that NBs elaborate angiogenic peptides. It has been reported previously that VEGF is expressed in human NB specimens (41, 42), but to our knowledge, no analysis of other important angiogenic factors has been performed thus far in NB. However, it is unlikely that a single angiogenic factor regulates the angiogenic process in any tumor system. Therefore, the purpose of this study was to determine the angiogenic profile of NB. Differential mRNA expression of a panel of angiogenic factors was examined in 37 primary tumors and 22 NB cell lines and compared with age, stage, histology, MYCN amplification, TrkA expression, and outcome.

**MATERIALS AND METHODS**

**NB Cell Lines and Tumor Samples.** All cell lines were obtained from the Children’s Hospital of Philadelphia cell line bank and grown at 5% CO₂ in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. Twenty-seven NB tumor samples were obtained from the Children’s Hospital of Philadelphia tumor bank, and 10 NB tumors were samples from the Quebec Neuroblastoma Screening Study.

**RNA Extraction and First-Strand cDNA Synthesis.** Total RNA was extracted either by using the RNeasy Kit (Qiagen, Valencia, CA) or according to the method of Chomczynski and Sacchi (43). Reactions were carried out using 1.0 μg of total RNA.
RNA in a total volume of 20 μl containing 150 ng of random hexamers (Life Technologies, Inc., Gaithersburg, MD), 0.5 mM dNTPs (Life Technologies, Inc.), 10 mM DTT, and 200 units of SuperScriptII reverse transcriptase (Life Technologies, Inc.) in the reaction buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂]. Initially, the total RNA was denatured at 70°C for 10 min and immediately chilled on ice. First-strand cDNAs were obtained after 10 min at 23°C and 50 min at 42°C. The reaction was terminated at 70°C for 15 min. RNase H (2 units; Life Technologies, Inc.) was added to each RT reaction followed by incubation at 37°C for 20 min.

**Semiquantitative RT-PCR.** PCR was carried out in a final volume of 10 μl containing 0.5 units of Taq Gold Polymerase, 200 μM dNTPs, and 0.4 μM of each primer in a buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM MgCl₂, and 1 μl of the RT product (reverse-transcribed total RNA)]. Specific PCR primers for VEGF, PDGF-A, TGF-α, and bFGF have been described previously (44) and were designed to bracket cDNA sequences that cross an intron-exon boundary in genomic DNA. Primer sequences for VEGF were able to detect two of four different molecular species produced by alternative splicing: mRNA VEGF₁₆₅ and VEGF₁₂₁. The expected PCR product size was 576 bp and 444 bp, respectively. Primers for VEGF-B were able to detect VEGF-B₁₆₇ with an expected PCR product size of 299 bp. Specific primer sequences for VEGF-B, PDGF-A (PCR product size 228 bp), TGF-α (PCR product size 241 bp), bFGF (PCR product size 238 bp), Ang-1 (PCR product size 263 bp), Ang-2 (PCR product size 202 bp), VEGF-C (PCR product size 228 bp), and glyceraldehyde-3-phosphate dehydrogenase (PCR product size, 160 bp) are available upon request. All PCR primers were biotinylated at their 5’ ends. PCR samples were overlayed with mineral oil, and amplification was performed on

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**Fig. 3** Expression of angiogenic peptides in primary NB tumors. The expression levels of VEGF₁₆₅/VEGF₁₂₁, VEGF-B, VEGF-C, bFGF, Ang-1, Ang-2, TGF-α, and PDGF-A transcripts in primary NB were determined by semiquantitative RT-PCR (as described in “Materials and Methods”). The expression of the target transcript was normalized by taking the ratio of the densitometric unit of the transcript:densitometric unit of the internal control, GAPD. The Y-axis represents the normalized expression level of the angiogenic agent, and numbers on the X-axis represent tumor stages. Differential expressions of angiogenic factors in favorable (stages 1, 2, and 4S) and unfavorable (stages 3 and 4) tumors were assessed by a two-sample t test on the mean expression values between the two groups. Differential expression was statistically significant, as indicated by the Ps.
Fig. 3. Continued
Biological Features. From the NB specimens in our study, 20 tumors had a favorable histology and 17 had an unfavorable histology. MYCN amplification was detected in 6 of 37 tumors (all of them stage 4 tumors), and 31 tumors had a single copy of the MYCN proto-oncogene. Sixteen of 22 NB cell lines were MYCN-amplified, and six cell lines had a single copy of MYCN.

Angiogenic Factors Are Expressed at Various Levels in Primary NBs and NB Cell Lines. We used semiquantitative RT-PCR analysis to examine the expression of eight angiogenic factors in NB. Fig. 1 demonstrates the expression of three angiogenic factors in six different NB cell lines as a representative example. Transcripts of VEGF-165 and VEGF-121 were detected in 36 and 35, respectively, of 37 tumors. All 37 tumors expressed various levels of VEGF-B, VEGF-C, Ang-2, and PDGF-A. Transcripts for bFGF were detected in 36 tumors, and transcripts for Ang-I and TGF-α were detected in 35 tumors. From the 22 NB cell lines examined, all 22 expressed various levels of VEGF-165, VEGF-121, VEGF-B, and bFGF (Fig. 2). Nineteen cell lines expressed PDGF. 18 cell lines expressed Ang-I and TGF-α, and 16 cell lines expressed transcripts of VEGF-C and Ang-2 (Fig. 2). Data obtained by RT-PCR were confirmed by a specific ELISA for VEGF and bFGF protein in supernatants of NB cell lines. Expression on mRNA and protein levels correlated well with each other (data not shown). However, no paraffin-embedded material for immunohistochemistry or protein material for Western blotting was available for the primary tumor specimens.

The Mean Expression Levels of Angiogenic Factors Are Significantly Higher in Advanced Stage NB. The mean expression levels of all eight angiogenic factors were higher in advanced-stage tumors (stages 3 and 4) than in low-stage tumors (stages 1, 2, and 4S; Fig. 3). This association was statistically significant for VEGF-165 (P < 0.0001), VEGF-121 (P = 0.0001), VEGF-B (P = 0.004), VEGF-C (P = 0.003), bFGF (P = 0.002), Ang-2 (P = 0.0001), TGF-α (P = 0.0007), and PDGF-A (P = 0.026; Table 1).

Correlation of VEGF Expression with Other Angiogenic Factors in NB Tumors. The level of expression of VEGF-165 was positively correlated with the expressions of VEGF-121, VEGF-B, VEGF-C, bFGF, Ang-1, and Ang-2 [correlation coefficient (r) = 0.5–0.76; P < 0.0001–0.002], but not with TGF-α and PDGF-A. The expression of VEGF-121 was also correlated with VEGF-B, VEGF-C, bFGF, Ang-1, and Ang-2 (r = 0.4–0.65; P < 0.0001–0.05). In addition, VEGF-B transcript levels were positively correlated with VEGF-C, bFGF, Ang-2, and TGF-α (r = 0.4–0.7; P < 0.0001–0.028), but not with Ang-1 and PDGF-A. VEGF-C expression was also correlated with Ang-2 and bFGF (r = 0.65; P < 0.0001), and bFGF was correlated with Ang-1 (r = 0.4; P = 0.014) and Ang-2 (r = 0.82; P < 0.0001). Ang-1 and Ang-2 were positively correlated with each other (r = 0.53; P = 0.0006). There was no correlation of PDGF-A expression with any other angiogenic factor examined. In NB cell lines, we found a significant correlation in the expression of VEGF-165, VEGF-121, VEGF-B, and bFGF (r = 0.45–0.92; P < 0.0001–0.05). TGF-α expression was positively correlated with Ang-1 (r = 0.55; P = 0.008) in NB cell lines. All other correlations were not significant in NB cell lines.

Correlation with TrkA Expression. Because the expression of angiogenic factors was found to be associated with advanced-stage tumors, we examined whether their expression was inversely associated with TrkA expression, a well-established prognostic marker of favorable NB (48–50). The expression of TrkA in our study cohort of NB examined with the same semiquantitative RT-PCR also served as a control for sampling bias (see “Discussion”). High TrkA expression was positively associated with better outcome and survival, younger age, lower stage, and favorable histology. We showed an inverse correlation of TrkA expression and expression of TGF-α in NB tumors ($r = -0.37; P = 0.02$) and cell lines ($r = -0.42; P = 0.05$). The correlation in expression of all other angiogenic factors with TrkA was not significant.

Correlation of TrkA and Angiogenic Factor Expression with Age, Histology, and MYCN Amplification. We detected an inverse correlation of TrkA expression and favorable histology ($P = 0.037$) and between higher age and unfavorable histology ($P = 0.006$). The mean expression levels of VEGF$_{123}$ ($P = 0.044$) and Ang-2 ($P = 0.012$) were significantly higher in tumors with unfavorable histology, whereas no other angiogenic factor showed any significant association with histology or age (Table 1).

In NB tumors, high TrkA expression ($P = 0.009$) and younger age ($P = 0.037$) were associated with single-copy MYCN. From the angiogenic factors, high expression of PDGF-A correlated significantly with MYCN amplification, but no other correlation of angiogenic factor expression and MYCN status was found in primary tumors. In NB cell lines, high expression of TrkA was associated with single-copy MYCN ($P = 0.023$). The mean expression level of VEGF-B was also higher in NB cell lines with single-copy MYCN ($P = 0.017$), whereas the mean expression levels of VEGF-C were higher in NB cell lines with MYCN amplification ($P = 0.0046$; Fig. 4). No other correlations with MYCN status were found to be significant in cell lines.

Association of Angiogenic Factor Expression with Outcome and Survival. Cox regression analysis revealed that high expression of TrkA was highly associated with event-free survival ($P = 0.007$) and overall survival ($P = 0.03$). Also, favorable histology was highly associated with overall survival ($P = 0.005$). From the angiogenic factors examined, only low-level expression of PDGF-A was significantly associated with overall survival of the patients ($P = 0.04$). However, there was also no significant association of age, stage, and MYCN amplification with survival.

DISCUSSION

Several studies have demonstrated that changes in the net balance of angiogenesis inhibitors and activators directly affect vascularity, tumor growth, and metastasis (51). Neovascularization has been shown to be associated with aggressive behavior in many adult malignancies (3, 5, 12–16, 40, 44). It has also been demonstrated previously that higher vascularity strongly correlates with disseminated disease and poor survival in NB (40). Because of the complex nature of the angiogenic process, it is unlikely that a single factor is responsible for angiogenesis in a particular tumor type. In NB, only expression of VEGF has been studied thus far (41, 42). To further explore the biological significance of angiogenesis in NB, we performed a systematic

### Table 1 Correlation of angiogenic factor expression with clinical and prognostic factors

<table>
<thead>
<tr>
<th></th>
<th>VEGF$_{165}$</th>
<th>VEGF$_{123}$</th>
<th>VEGF-B</th>
<th>VEGF-C</th>
<th>Ang-1</th>
<th>Ang-2</th>
<th>bFGF</th>
<th>TGF-α</th>
<th>PDGF</th>
<th>TrkA</th>
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<td>Stage</td>
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<td>$0.0001$</td>
<td>$0.004$</td>
<td>$0.003$</td>
<td>n.s.</td>
<td>$&lt;0.0001$</td>
<td>$0.002$</td>
<td>$0.0007$</td>
<td>$0.026$</td>
<td>$0.0002$</td>
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<td>n.s.</td>
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<td>n.s.</td>
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<tr>
<td>Age</td>
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<td>n.s.</td>
<td>0.05</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>0.038</td>
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<td>MYCN</td>
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<td>n.s.</td>
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<td>n.s.</td>
<td>n.s.</td>
<td>$0.002$</td>
<td>$0.0009^b$</td>
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<tr>
<td>Histology</td>
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<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>$0.012$</td>
<td>n.s.</td>
<td>n.s.</td>
<td>$0.023^b$</td>
<td>n.s.</td>
<td>0.037</td>
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<tr>
<td>TrkA</td>
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<td>n.s.</td>
<td>n.s.</td>
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* EFS, event-free survival; n.s., not significant.

$^b$ Inverse correlation.
analysis of expression of angiogenic factors in NB cell lines and tumor samples. The tumor tissue and cell line data obtained in our study correlated well with each other. However, data obtained using tumor tissue might be more reliable because cell lines are an artificial system in some respects and are subject to selection of cells with more aggressive features that are able to survive in vitro under cell culture conditions.

Our results indicate that all eight angiogenic factors examined are present at various levels in NB cell lines and tumors, suggesting their involvement in NB angiogenesis. We found that high expression levels of seven angiogenic factors (VEGF, VEGF-B, VEGF-C, bFGF, Ang-2, TGF-α, and PDGF-A) correlated strongly with the advanced stage of NB. In the study by Roessler et al. (41), VEGF immunoreactivity in 10 NB tumor samples did not correlate with clinical stage. This might be attributable to the lower number of tumor specimens in that study, but might also suggest that RT-PCR is a more sensitive method than immunohistochemistry for the quantification of angiogenic factor expression in NB.

We also examined whether the expression of angiogenic factors was associated with well-established favorable and unfavorable prognostic markers of NB, i.e., TrkA expression and MYCN amplification, respectively. The examination of TrkA expression by the same RT-PCR method also served as a control for method reliability and sampling bias because TrkA expression has been investigated in large study cohorts, and its expression pattern in NB has been well established (48–50). In fact, our data on TrkA expression were consistent with the previous findings that high TrkA expression was associated with low-stage, favorable NB (P = 0.0002; data not shown). These and other data suggest that our study cohort is generally representative of an unselected population of these patients. Thus, the expression pattern of the angiogenic factors examined is likely to reflect that in the general NB population.

We demonstrated in our study that the expression of PDGF-A correlates significantly with overall survival of the patients, but no other angiogenic factor was found to be associated with outcome or survival. However, future studies in larger study cohorts will be required to determine whether the expression of angiogenic factors is predictive of NB outcome and might serve as an independent prognostic factor.

The fact that the expression level of most angiogenic factors is not associated with MYCN amplification (except for an inverse correlation with PDGF-A) in NB tumors and that only TGF-α expression is inversely correlated with TrkA expression might also be attributable to the low number of patients in our study. However, it is interesting that VEGF-B is correlated with single-copy MYCN in NB cell lines, whereas VEGF-C is highly associated with MYCN amplification. One might speculate that up-regulation of VEGF-C is a mechanism used by MYCN-amplified, aggressive NB tumors to attract not only vascular endothelial cells, but also lymphatic endothelial cells. Expression of VEGF-C is associated with the development of lymphatic vessels. Although angiogenesis refers to an increase in blood vessel formation, others have found that angiogenesis is also associated with increased lymph node metastasis (52, 53). Angiogenesis of lymphatic vessels might be a process similar to vascular angiogenesis, providing a subset of tumors with the opportunity of metastatic spread via the lymphatics. VEGF-C could be an important factor regulating paracrine relationships between tumor cells and lymphatic endothelial cells (28). At present, it is unclear if high levels of MYCN expression regulate this process at least in part by up-regulation of VEGF-C. Whether VEGF-C expression is also associated with MYCN amplification in primary NB tumors has to be determined in a larger study cohort with more MYCN-amplified tumors. A possible relationship between MYCN amplification and VEGF-C up-regulation is in agreement with previous studies demonstrating that NB angiogenesis correlates with MYCN amplification and metastatic disease (40) and that enhanced MYCN expression induces angiogenesis of experimental human NBs (54).

Most angiogenic factors were expressed at relatively low levels in the four 4S tumors in our study. In contrast, in the report by Meitar et al. (40), three 4S tumors were highly vascular, which might be expected because of the metastatic disease that 4S patients demonstrate at diagnosis. However, unlike stage 4 tumors, it is well known that the outcome for this special stage of disseminated NB is generally favorable because these 4S tumors have a propensity to undergo spontaneous remission. Although stage 4S tumors might be widely metastatic because they initially have an angiogenic phenotype, the unique biological features of this special subset of NB appear to alter this phenotype. Thus, the time point of diagnosis (before or after initiation of regression) might be important for the determination of angiogenic factor expression.

Expression levels of VEGF, VEGF-B, VEGF-C, bFGF, Ang-1, and Ang-2 are correlated with each other in NB specimens. This suggests that several angiogenic peptides act in concert in the regulation of neovascularization. Up-regulation of VEGF family members might be mediated by up-regulation of common transcription factors, or some angiogenic factors may act through a second messenger system by inducing the expression of other angiogenic factors. VEGF-related factors may interact with the VEGF system in a number of ways, e.g., VEGF-B is known to form heterodimers with VEGF (24). The existence of a gene family consisting of several related growth factors suggests that these family members have overlapping but distinct functions. A synergistic effect of bFGF and VEGF has also been reported before (55). TGF-α was found to function as a potent inducer of VEGF synthesis by transcription of the VEGF gene promoter via AP2 transcription factors (36). However, we did not find a statistically significant correlation between expression of TGF-α and VEGF in our study, suggesting that interaction of VEGF with other angiogenic factors may be more important.

Taken together, our results suggest that several angiogenic factors have a biological role in NB angiogenesis. They might contribute synergistically to a more aggressive unfavorable tumor biology. The ubiquitous expression of several angiogenesis stimulators in NB suggests that antiangiogenesis therapy may provide a novel strategy that may be particularly useful for highly vascularized, advanced-stage tumors. However, the redundant expression of other angiogenic factors also suggests that molecules targeting only VEGF and inhibiting its bioactivity selectively might not be sufficient as antiangiogenic agents in NB. More general antiangiogenic approaches may be necessary, like therapy with the angiogenesis inhibitor TNP-470, a synthetic angiotastic agent derived from Aspergillus fumigatus.
This agent specifically inhibits endothelial proliferation independent of angiogenic factor expression, and so it might be more promising (56).

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