Treatment of Experimental Neuroblastoma with Angiogenic Inhibitors

BY
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Abstract

Neuroblastoma is a childhood cancer that originates from neuroblasts in the peripheral nervous system. Neuroblastoma show considerable heterogeneity with respect to location, responsiveness to treatment and prognosis. Since current therapy involves drugs with risk of serious side effects in the growing child, there is a clinical need for more effective and less toxic treatment strategies.

Angiogenesis, the formation of new blood vessels, is critical for tumor progression. Specific inhibition of tumor-induced angiogenesis should restrict growth of most solid tumors and thereby provide a new treatment strategy. The aim of this study was to investigate the effects of angiogenic inhibition in experimental neuroblastoma in mice.

We found that experimental neuroblastomas expressed the perhaps most potent angiogenic growth factor, VEGF-A, and that plasma VEGF-A levels correlated with tumor size. SU5416, a novel antagonist of VEGFR-1 and 2, reduced angiogenesis and tumor growth in our model.

We also investigated the properties of SU11657, a new, orally available, synthetic small molecule multi-targeted tyrosine kinase inhibitor. SU11657, at a well-tolerated dose, was more potent than SU5416 in reducing tumor growth rate and angiogenesis, even in MYCN-amplified tumors. Chemotherapeutics can also inhibit angiogenesis, when administrated daily in a non-toxic dose. CHS 828, a new chemotherapeutic, given orally, alone induced complete neuroblastoma regression in 44 % of the animals. Furthermore, the bisphosphonate zoledronic acid, developed to reduce bone resorption, showed anti-tumor activity in our model. Zoledronic acid was more potent than the angiogenic inhibitor TNP-470. Thus bisphosphonates may have other beneficial properties in patients with cancer apart from preventing bone resorption.

In conclusion, SU5416, SU11657, CHS 828, and zoledronic acid represent new drugs with potent anti-tumor effects. Angiogenic inhibition as single therapy or in combination with chemotherapeutics may be beneficial in the treatment of rapidly growing and highly vascularized solid tumors of childhood such as neuroblastoma.

Keywords: Angiogenesis, Neuroblastoma, MYCN, VEGF, SU11657, SU5416, TNP-470, zoledronic acid

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To my family
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CgA</td>
<td>Chromogranine A</td>
</tr>
<tr>
<td>c-KIT</td>
<td>Stem cell factor receptor</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FL</td>
<td>FLT-3 ligand</td>
</tr>
<tr>
<td>FLT3</td>
<td>Fms-related tyrosine kinase 3</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>NRS</td>
<td>Normal rabbit serum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium chloride-sodium citrate buffer</td>
</tr>
<tr>
<td>T/C</td>
<td>Mean tumor of treated tumors/mean volume of control tumors</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP-biotin nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
</tbody>
</table>
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


II. Bäckman U., and Christofferson R. The Selective Class III/V Receptor Tyrosine Kinase Inhibitor SU11657 Inhibits Tumor Growth and Angiogenesis in Experimental Neuroblastomas Grown in Mice. *Submitted for publication*.


IV. Bäckman U., Svensson Å., and Christofferson R. The Bisphosphonate Zoledronic acid Reduces Experimental Neuroblastoma Growth by Interfering with Tumor Angiogenesis. *Submitted for publication*.

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Introduction

Childhood cancer

Ten percent of all deaths during childhood are related to cancer, making this the most common cause of death from disease. In Sweden, the annual incidence of childhood malignancies is approximately 15 cases per 100,000 children as recorded by the Nordic Society of Paediatric Haematology and Oncology (NOPHO, Gustafsson et al. 1999). Among 12 major types of childhood cancers, leukemias, brain tumors, and other tumors of the central nervous system account for over one-half of the new cases. The causes of childhood cancers are largely unknown, and Down syndrome, other specific chromosomal and genetic abnormalities, and exposure to ionizing radiation can explain only a small percentage of the cases (Narod et al. 1991).

Chemotherapy, and when applicable, surgery and radiation therapy are the cornerstones of treatment of childhood cancers. The principles of pediatric chemotherapy have evolved from clinical experience, including the use of combination therapies, adjuvant therapies, and the administration of drugs at a maximum tolerated dose (MTD) according to specific treatment protocols. Both acute toxicity and late adverse effects are of great concern. The acute toxicity involves myelosuppression, nausea and vomiting, alopecia, orointestinal mucositis, liver function test abnormalities, allergic reactions, diarrhea, fever, and pain. Since children grow and have a long expected life span, the risk of late adverse effects of treatment such as retardation of growth and development, infertility, cardiac, pulmonary, or renal impairment, and possible carcinogenic and teratogenic effects must be considered. The late effects depend on the chemotherapy given, the dose, and the age of the child during therapy. Today three out of four children are cured, but there are malignancies such as neuroblastoma, medulloblastoma, osteosarcoma, and Ewing’s sarcoma where the survival is below 60 % (Gustafsson et al. 2000).
Neuroblastoma

Neuroblastoma is the most common of the extracranial solid tumors in children. Neuroblastoma is staged clinically according to the international neuroblastoma staging system (INSS) (Table 1). The tumors in neuroblastoma arise from the adrenal gland, the sympathetic cord or paraganglia and are located in the thoracic or abdominal cavities. Sometimes, but less frequently, the tumors are found in the neck, head or pelvis (Maris et al. 1999, Evans et al. 1987). Their biological behavior is intriguing; some tumors regress spontaneously, whereas others progress despite aggressive multimodality therapy.

Table 1. Staging system for neuroblastoma according to the international neuroblastoma staging system (INSS)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>Localized tumor confined to the area of origin; complete gross excision, with or without microscopic residual disease; identifiable ipsilateral and contralateral lymph nodes negative for tumor microscopy.</td>
</tr>
<tr>
<td>Stage 2A</td>
<td>Unilateral tumor with incomplete gross excision; identifiable ipsilateral and contralateral lymph nodes negative for tumor microscopy.</td>
</tr>
<tr>
<td>Stage 2B</td>
<td>Unilateral tumor with complete or incomplete gross excision; with positive ipsilateral regional lymph nodes; identifiable contralateral lymph nodes negative for tumor microscopy.</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Tumor infiltrating across the midline with or without regional lymph node involvement; or unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral lymph node involvement.</td>
</tr>
<tr>
<td>Stage 4</td>
<td>Dissemination of tumor to distant lymph nodes, bone, bone marrow, liver, or other organs.</td>
</tr>
<tr>
<td>Stage 4S</td>
<td>Stage 1 or 2 otherwise in children aged &lt; 1 year, with metastases in liver, skin, bone marrow but not in the bone.</td>
</tr>
</tbody>
</table>

(Brodeur et al. 1988, Hayes et al. 1989)
Patient age above one year, advanced tumor stage, and amplification of the MYCN oncogene are three established negative prognostic factors that influence survival (Komuro et al. 2001).

Neuroblastoma is the third most frequent form of cancer and accounts for approximately 5-7% of all malignancies in children. Of these children 25% are under 1 year of age at diagnosis and 75% of all children with neuroblastoma are under 5 years old (Brodeur et al. 1997). In Sweden, the annual incidence is 15-20 new cases (age 15 years or less) (Gustafsson et al. 1999). The overall 5-year survival in Europe is about 45% (Cotterill et al. 2000). In the majority of cases (73%), neuroblastoma has already spread to areas outside the original site at the time of diagnosis. Diagnosis of neuroblastoma can be complicated, but in 90% of cases of neuroblastoma, elevated levels of urinary catecholamine metabolites are found. The survival of patients with advanced disease and dismal prognostic markers is still poor. In view of the long expected life span of survivors of childhood cancer, this underlies the importance of finding new treatment strategies, based, for instance, on inhibition of tumor angiogenesis.

Angiogenesis

Angiogenesis, the process by which capillaries sprout from pre-existing blood vessels, is a complex event involving several factors on the molecular and cellular levels, with both stimulating and inhibiting steps. When initiated, angiogenesis is characterized by a rapid cascade of events involving processes such as dissolution of vascular basal membranes, increased vascular permeability, and degradation of the extracellular matrix. Interactions between the vascular endothelium and the extracellular matrix are required for endothelial cell migration and tube formation (Fox et al. 2001). These interactions are mediated by integrins such as αvβ3 and αvβ5 (Ellis et al. 2001a). There is accumulating evidence that blood vessels in tumors are structurally and functionally abnormal. The pericytes on tumor vessels are abnormal in shape, express atypical markers, and have a loose association with endothelial cells (Morikawa et al. 2002). The tumor vessel walls have numerous openings through endothelial fenestrae, vesicles and transcellular holes, as well as widened interendothelial junctions. These defects make tumor vessels hyperpermeable and leaky (Dvorak et al. 1988, Jain et al. 1996, Hobbs et al. 1998, Feng et al. 2000).
A tumor needs to establish vasculature to obtain the metabolic exchange necessary to sustain growth beyond 1-2 mm$^3$ (Liotta et al. 1974, Zetter et al. 1998). Epigenetic changes in the tumor environment, such as hypercapnia, hypoxia, and nitric oxide production, and genetic events such as inhibition of tumor suppressor genes may trigger the ingrowth of new vessels (Ellis et al. 2001b). Not until the tumor is vascularized can metastatic spread occur. Tumor angiogenesis is hence a vital prerequisite for the progression of a cancer in situ to an expanding tumor with metastatic spread (Folkman, 1971, Liotta et al. 1974).

Angiogenic factors

More than 17 angiogenic growth factors and their corresponding membrane receptors have been identified (Table 2), i.e. vascular endothelial growth factor (VEGF) (Ferrara et al. 1989), acidic/basic fibroblast growth factor (aFGF, bFGF) (Rifkin et al. 1989, Jouanneau et al. 1995), platelet-derived growth factor (PDGF) (Nicosia et al. 1994), platelet-derived endothelial cell growth factor (PD-ECGF) (Takahashi et al. 1996), hepatocyte growth factor (HGF) (Merkulova-Rainen et al. 2003), angiopoietin-1 (Suri et al. 1998), angiogenin (Bruserud et al. 2003), interleukin-8 (IL-8) (Ferrer et al. 2000), transforming growth factor β (TGF-β) (Pepper et al. 1993), transforming
growth factor α (TGF-α) (Gleave et al. 1993), epidermal growth factor (EGF) (Ferrara et al. 1989), Substance P (SP) (Thurston et al. 1998), hematopoietic cytokines (Rosolen et al. 1997), granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF) (Ninck et al. 2003). Apart from these peptides, certain physiological factors (e.g. hypoxia) as well as small molecules (e.g. hydroxybutyrate) are known to induce angiogenesis.

Table 2. Angiogenic growth factors – peptides that stimulate angiogenesis in vivo and in vitro.

<table>
<thead>
<tr>
<th>Acute fibroblast growth factor</th>
<th>aFGF, FGF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenin</td>
<td>ANG</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td>ANG-1</td>
</tr>
<tr>
<td>Basic fibroblast growth factor</td>
<td>bFGF, FGF2</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>EGF</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>EPO</td>
</tr>
<tr>
<td>Granulocyte/macrophage-colony stimulating factor</td>
<td>GM-CSF, G-CSF</td>
</tr>
<tr>
<td>Hepatocyte growth factor</td>
<td>HGF</td>
</tr>
<tr>
<td>Interleukin-8</td>
<td>IL-8</td>
</tr>
<tr>
<td>Leptin</td>
<td>NPY</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td></td>
</tr>
<tr>
<td>Platelet-derived growth factor</td>
<td>PDGF</td>
</tr>
<tr>
<td>Platelet-derived endothelial cell growth factor</td>
<td>PD-ECGF</td>
</tr>
<tr>
<td>Substance P</td>
<td>SP</td>
</tr>
<tr>
<td>Transforming growth factor alpha/beta</td>
<td>TGF-α/β</td>
</tr>
<tr>
<td>Tumor necrosis factor alpha</td>
<td>NF-α</td>
</tr>
<tr>
<td>Vascular endothelial growth factor-A</td>
<td>VEGF-A</td>
</tr>
</tbody>
</table>

Vascular Endothelial Growth Factor

As stated above, the formation of new blood vessels is complex and is tightly regulated. Among the most important factors in the induction and maintenance of new capillaries are the members of the VEGF family. VEGF is a 46 kDa homodimeric glycoprotein produced by tumor and stromal cells in response to hypoxia and other stimuli (Folkman, 1971, Ellis et al. 2000) (Table 3) that is up-regulated under hypoxic conditions and down-regulated under hyperoxic conditions (Shweiki et al. 1992, Alon et al. 1995). The most potent and well-studied angiogenic factor is VEGF-A. This has an ability to promote endothelial proliferation and to induce leakiness of blood vessels (Connolly et al. 1989, Dvorak et al. 1995, Ferrara et al. 1993, Senger et al. 1983). Alternative exon splicing of VEGF-A was initially shown to
result in four different isoforms VEGF_{121}, VEGF_{165}, VEGF_{189}, and VEGF_{206} (Houck et al. 1991, Tischer et al. 1991), of which the predominant isoforms are VEGF-A_{165} and VEGF-A_{189} (Robinson and Stringer 2001). Less frequent splice variants have been also reported, such as VEGF_{145} and VEGF_{183} (Neufeldt et al. 1999). VEGF-A_{121}, 145, 165, and 183 are secreted whereas VEGF-A_{189} and 206 are membrane-bound and not diffusible. VEGF-A is expressed in many tissues, both in adults and during fetal life, and it is also expressed by most tumors. The biological importance of VEGF-A is evident from the observation that knock-out of one single allele is lethal (Ferrara et al. 1996, Carmeliet et al. 1996). The VEGF family includes five more members: placental growth factor (PIGF) (Maglione et al. 1991), VEGF-B (Grimmond et al. 1996, Olofsson et al. 1996), VEGF-C (Kukk et al. 1996), VEGF-D (Juokov et al. 1996, Lee et al. 1996), and VEGF-E (Lyttle et al. 1994).

**Table 3. VEGF research milestones**

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1971</td>
<td>Folkman published angiogenic hypothesis (Folkman, 1971)</td>
</tr>
<tr>
<td>1983</td>
<td>Dvorak showed that tumors secrete a vascular permeability factor (Senger et al. 1983).</td>
</tr>
<tr>
<td>1989</td>
<td>Ferrara purified and cloned vascular endothelial growth factor (Ferrara et al. 1989).</td>
</tr>
<tr>
<td>1997</td>
<td>First clinical trials of specific VEGF inhibition in cancer patients began.</td>
</tr>
<tr>
<td>2003</td>
<td>Phase III colorectal cancer trial indicate prolonged survival (Avastin – anti-VEGF- ab) (Fernando et al. 2003).</td>
</tr>
</tbody>
</table>

VEGF receptors

Growth factors exert their action by binding to specific surface receptors, which thereby transduce the growth factor signal into the cell. VEGF-A binds two related receptor tyrosine kinases VEGFR-1 and VEGFR-2. The VEGF receptors have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain (Shibuya et al. 1990, Terman et al. 1991). The dimeric form binds two receptors simultaneously, and thus forming a stable dimer that becomes activated by autophosphorylation.
VEGFR-1 or Flt-1 (fms-like tyrosine kinase-1), was the first receptor tyrosine kinase to be identified as a VEGF receptor a decade ago (de Vries et al. 1992). VEGFR-1 binds not only VEGF-A but also PlGF (Park et al. 1994) and VEGF-B (Olofsson et al. 1998) (Figure 2). The VEGFR-1 gene gives rise to two different isoforms: a full-length receptor and an extracellular form called soluble VEGFR-1 (sVEGFR-1) (Kendall et al. 1996).

Figure 2. Representation of the interactions between VEGF ligands and receptors.

VEGFR-2, the second receptor found, was originally called KDR (kinase insert domain-containing receptor) for the human protein and Flk-1 (fetal liver kinase 1) for the mouse homologue (Matthews et al. 1991, Terman et al. 1991). VEGFR-1 and VEGFR-2 are expressed almost exclusively on endothelial cells, but can also be expressed on some VEGF-expressing tumor cells, creating the possibility of an autocrine loop (Masood et al. 1997, de Jong et al. 1998). VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF. Signaling through VEGFR-2 stimulates capillary dilation and permeabilization endothelial cell
proliferation, migration, protease release, and survival through activation of multiple signal cascades (Hubbarb et al. 1999, Gerber et al. 1999). VEGFR-3/Flt-4 is a member of the same family of receptor tyrosine kinases but is not a receptor for VEGF-A, binding instead to VEGF-C and VEGF-D (Karkkainen et al. 2002). In addition to these receptors, VEGF interacts with a family of coreceptors, the neuropilins. Neuropilins (NP-1) modulate the interaction of VEGF with VEGFR and enhances the binding of VEGF-A165 to VEGFR-2 (Soker et al. 1998). The closely related NP-2 also functions as a receptor for VEGF-A145 and VEGF-A165 (Gluzman-Poltorak et al. 2000). Increased expression of VEGF and its receptors has been observed in many human solid tumors (Droller et al. 1998, Shaheen et al. 1999, Yoshiji et al. 1999, Balbay et al. 1999).

Platelet Derived Growth Factor
PDGF has important effects on many cell types including, endothelial cells in vivo (Betsholz et al. 1995). The PDGF family is encoded by two genes (PDGF-A and PDGF-B), resulting in three dimeric isoforms of protein, namely PDGF-AA, PDGF-BB, and PDGF-AB (Heldin et al. 1979, Johnson et al. 1982, Waterfield et al. 1983). Both the A- and B- chain contains a carboxy terminal retention motif that keeps the secreted protein on the cell surface or in the endothelial cell membrane. PDGF-B is diffusible while the gene for PDGF-A encodes for two splice isoforms- one long, cell surface-restricted form and one freely diffusible form, lacking the carboxy-terminus (Heldin and Westermark 1999). PDGF isoforms are reported to exert mitogenic and chemotactic actions on endothelial cells (Edelberg et al. 1998, Nicosia et al. 1994, Risau et al. 1992). Results show that PDGF-BB consistently induces an angiogenic response (Risau et al. 1992), and modulates endothelial proliferation and angiogenesis (Battegay et al. 1994). It has also been shown that targeted inactivation of PDGF-A, PDGF-B, or their receptors leads to embryonic death (Boström et al. 1996). In addition two new members of the PDGF family have been discovered, PDGF-C and PDGF-D (Li et al. 2000, Bergsten et al. 2001).

PDGF receptors
PDGFs interact with different affinities with three tyrosine kinase receptors, PDGFR-αα, PDGFR-ββ, and PDGFR-αβ (Heldin et al. 1998, Lindahl et al. 1998, Kazlauskas et al. 2000), which are expressed on endothelial cells under both normal (Marx et al. 1994, Edelberg et al. 1998, Takase et al. 1999) and pathological conditions (Ebert et al. 1995, Lemström et al. 1997, Peschen et al. 1998, de Jong et al. 1998). These receptors are structurally related to VEGF receptors, but contain five extracellular Ig domains instead
of seven. PDGFR-αα binds PDGF-AA, PDGF-BB, PDGF-AB and PDGF-CC and PDGFR-ββ binds PDGF-BB and PDGF-DD and PDGFR-αβ binds PDGF-BB and PDGF-AB (Figure 3). A majority of cell types express only one isoform (α or β). Capillary endothelial cells express PDGFR-β and are stimulated by PDGF-BB not only to increase DNA synthesis (Bar et al. 1989, Battegay et al. 1994) but also to form angiogenic sprouts in vitro (Battegay et al. 1994, Nicosia et al. 1994). In addition, PDGF stimulates the proliferation of cultured smooth muscle cells and pericytes (Edelberg et al. 1998), both of which have been shown to express PDGFR-β (Heldin and Westermark, 1999).

**Figure 3. Representation of the interactions between PDGF ligands and receptors.**

Stem cell factor (SCF) and its receptor c-KIT

SCF is a secreted and membrane-bound glycoprotein dimer that plays a role in hematopoiesis, melanogenesis, and gametogenesis. Several types of cells, including endothelial cells, express SCF (Heinrich et al. 1993). SCF and its receptor c-KIT (Yarden et al. 1987, Zsebo et al. 1990) play an important role in the development of the peripheral nervous system and are expressed in neural crest-derived tumor cells such as those of neuroblastoma (Cohen et al. 1994). Autocrine SCF-c-KIT signal transduction pathways have been found to regulate the growth of neuroblastomas and glioblastomas (Berdel et al. 1999).
A recent study indicated that the main role of SCF in neuroblastomas could be that of protecting cells from apoptosis (Timeus et al. 1997). The possibility of a direct effect of SCF on tumor angiogenesis has not been examined, but it is hypothesized that SCF released from tumor cells attracts mast cells and hence leads to accelerated angiogenesis (Zhang et al. 2000).

FLT3 ligand (FL) and its receptor FLT3
FMS-related tyrosine kinase-3 (FLT3) is a transmembrane glycoprotein and is expressed on several different cell types (Birg et al. 1992, Drexler et al. 1999), including neuroblastoma cells (Timeus et al. 2001). FLT3 is structurally related to macrophage colony-stimulating factor receptor (CSF-1) and c-KIT. Its ligand, FLT3 ligand (FL) is a cytokine that promotes the proliferation, survival, and differentiation of hematopoietic progenitors (Zeigler et al. 1994, Brxmeyer et al. 1995, Rasko et al. 1995) in synergy with other growth factors, namely SCF, IL-3, IL-6, IL-12 and GM-CSF (Drouet et al. 1999). In association with G-CSF, FL mobilizes stem and progenitor cells to peripheral blood (Molineux et al. 1997, Papayannopoulou et al. 1997). FLT3 plays an important role in lymphopoiesis and in the progression of dendritic cells (Hunte et al. 1996, Ray et al. 1996, Ohm et al. 1999).

Therapeutic interference with VEGF/PDGF-induced angiogenesis
Since the formation of solid tumors is angiogenesis-dependent, several strategies have been developed for targeting the VEGF pathway as part of anticancer therapy (Kim et al. 1993, Prewett et al. 1999, Fong et al. 1999). The precise action of some of these compounds is not known, although their property of angiogenic inhibition has been demonstrated in vitro and in vivo. One strategy for interfering with VEGF/PDGF-induced angiogenesis is to use immunoneutralizing antibodies against VEGF/PDGF-receptor complexes. Another strategy, however, and perhaps the most promising approach to achieving growth factor receptor inhibition, is the use of tyrosine kinase inhibitors (Druker and Lydon 2000, Shawver et al. 2002). These low molecular weight compounds act by competing for the ATP-binding pocket in the kinase domain, thus effectively abolishing the transphosphorylation activity of the kinase. A number of selective compounds inhibiting the VEGF/PDGF receptor kinases have been synthesized, screened in vitro and successfully tested in various disease models. These include SU5416 (Fong et al. 1999) (Figure 4), SU6668 (Laird et al. 2000), PTK787 (Wood et al. 2000), and SU11657 (Pollard et al. 2002,
Sohal et al. 2003). SU11657 also inhibits c-KIT and FLT3. Yet other strategies involve the use of neutralizing antibodies against VEGF/PDGF, against dominant negative receptors that block the activity of VEGF/PDGF, and against soluble VEGF/PDGF receptors; and agents that prevent the release or activation of VEGF/PDGF. Administration of endogenous angiogenic inhibitors such as angioatin and endostatin, and gene transfer of DNA that encodes for angiogenic inhibitors, including angioatin and platelet factor 4, are also under evaluation (Rosen, 2000).

**Figure 4.** a) VEGF-induced angiogenesis, b) inhibition of the VEGF-receptor signaling pathway by SU5416.

Angiogenic inhibitors and chemotherapy

Unlike chemotherapy, which targets tumor cells and other proliferating cells, angiogenic inhibitors target activated endothelial cells (Table 4). Thus angiogenic inhibitors are not as likely to cause toxicities such as bone marrow depression and enteritis that are characteristic of chemotherapeutic regimens. In addition, acquired drug resistance is a major problem with chemotherapy, since most cancer cells are genetically unstable and are prone to mutations. In contrast, endothelial cells are less likely to develop resistance to angiogenic inhibitors, since these cells are untransformed and have a stable genome. Since angiogenic inhibitors are expected to be cytostatic rather than cytotoxic and since the strategies are directed at
different cellular targets, angiogenic combined with standard chemotherapy directed at tumors may prove useful (Rosen, 2000).

<table>
<thead>
<tr>
<th>Table 4. Angiogenic inhibitors compared with conventional chemotherapy.</th>
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<tbody>
<tr>
<td><strong>Target</strong></td>
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<tr>
<td>Endothelial cells</td>
</tr>
<tr>
<td><strong>Effect</strong></td>
</tr>
<tr>
<td><strong>Side effects</strong></td>
</tr>
<tr>
<td><strong>Dose scheduling</strong></td>
</tr>
<tr>
<td><strong>Drug resistance</strong></td>
</tr>
<tr>
<td><strong>Anti-tumor activity</strong></td>
</tr>
</tbody>
</table>
Present investigation

The fact that VEGF/PDGF and their receptor systems are considered to be fundamental regulators of blood vessel formation, made it important to address their suppression in pathological angiogenesis. By using reproducible mouse models for neuroblastoma, based on xenotransplantation of SH-SY5Y, SK-N-AS and IMR-32 neuroblastoma cells, we set out to investigate the effects of

- one well characterized angiogenic inhibitor, TNP-470 (paper IV)
- two new angiogenic inhibitors, SU5416 (VEGFR-1, 2 inhibition) (paper I), and SU11657 (VEGFR, PDGFR, c-KIT, and FLT3 inhibition) (paper II)
- a new cytotoxic drug, CHS 828, alone and in combination with TNP-470 and SU5416 (paper III)
- a new putative angiogenic inhibitor, zoledronic acid (paper IV)

on experimental neuroblastoma tumors.
Material and methods

Substances

All substances were dissolved in their solvent immediately before injection. **SU5416**, 3-[(2,4-dimethylpyrrol-5-yl) methylidenyl]-indolin-2-one (SUGEN Inc., South San Francisco, CA), was suspended in 0.5% (w/v) carboxymethylcellulose sodium, 0.9% (w/v) sodium chloride, 0.4% (v/v) polysorbate 80 and 0.9% (v/v) benzyl alcohol in deionized water to the appropriate concentration. **TNP-470** (Takeda Chemical Industries Ltd., Osaka, Japan) was suspended in 1% ethanol and 5% gum arabic in 0.9% sodium chloride. **CHS 828** (N- (6-chlorophenoxyhexyl)-N’-cyano-N”-4-pyridylguanidine (Leo Pharmaceutical Products, Ballerup, Denmark) was
suspended in peanut oil and **zoledronic acid** (Novartis Pharma AG, Basel, Switzerland) was suspended in phosphate-buffered saline, pH 6.5, in a calcium-free environment. **SU11657** (SUGEN Inc.) was suspended in 0.5 % (w/v) carboxymethylcellulose sodium (medium grade), 0.9 % (w/v) sodium chloride, 0.4 % (w/v) polysorbate 80 and 0.9 % (w/v) benzyl alcohol in deionized water to the appropriate concentration.

**Neuroblastoma cells**

We have been tested several neuroblastoma cell lines in nude mice i.e., the human neuroblastoma cells SK-N-SH, SH-SY5Y, SK-N-AS, SK-N-DZ, KELLY, IMR-32 and one mouse neuroblastoma cell line, Neuro-2a. On the basis of our results, i.e. tumor take and days from xenograft to tumor take/start of the study, we considered that SH-SY5Y, SK-N-AS and IMR-32 were the best cell lines to work with in our xenograft model (Table 5).

**Table 5. Tumor take for different neuroblastoma cell lines, 30 x 10⁶ cells injected s.c. in the right hindleg in NMRI nu-nu mice.** SK-N-SH, SH-SY5Y, SK-N-AS, IMR-32, SK-N-DZ, and KELLY are human neuroblastoma cell lines. Neuro-2a is a mouse neuroblastoma cell line. T= tumors and TV= tumor volume.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage</th>
<th>Days fr. to palpable T i.e. TV~ 0.1 ml</th>
<th>Days fr. to start, i.e. TV 0.3 ml</th>
<th>Days fr. start to termination of the study, i.e. TV ~ 3-4 ml</th>
<th>No. of T/ mice injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-SH</td>
<td>40</td>
<td>10-14</td>
<td>12-20</td>
<td>20-22</td>
<td>20 %</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>32</td>
<td>10-14</td>
<td>12-28</td>
<td>20-22</td>
<td>89 %</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>80</td>
<td>7-9</td>
<td>9-11</td>
<td>8-10</td>
<td>100 %</td>
</tr>
<tr>
<td>IMR-32</td>
<td>64</td>
<td>30-35</td>
<td>35-50</td>
<td>20-24</td>
<td>91 %</td>
</tr>
<tr>
<td>SK-N-DZ</td>
<td>80</td>
<td>21-25</td>
<td>25-32</td>
<td>10-15</td>
<td>25 %</td>
</tr>
<tr>
<td>KELLY</td>
<td>16</td>
<td>32-43</td>
<td>43-50</td>
<td>20-25</td>
<td>62 %</td>
</tr>
<tr>
<td>Neuro-2a</td>
<td>?</td>
<td>4-5</td>
<td>5-7</td>
<td>5-7</td>
<td>100 %</td>
</tr>
</tbody>
</table>

SH-SY5Y was kindly provided by Dr. June Biedler, The Memorial Sloan-Kettering Cancer Center, New York, USA. SK-N-AS and the MYCN-amplified IMR-32 were purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in Eagle’s minimum essential medium (SVA, Uppsala, Sweden) supplemented with 10 % fetal calf serum, 1mM glutamine, penicillin (100 IU/ml) and streptomycin (50
µg/ml) (Sigma Chemical Co., St Louis, MO). For SK-N-AS and IMR-32, 1 % non-essential amino acids were added to the medium (Sigma). The cells were grown in humidified air (95 %) and 5 % CO₂ at 37 °C. The medium was changed twice a week and confluent cultures were subcultivated after 5 minutes of treatment with 0.25 % trypsin and 0.02 % EDTA. For s.c. injections, a single cell suspension was prepared, where cells were resuspended in medium and the viability and cell concentration were calculated after addition of trypan blue dye. The cell suspension was kept on ice during the injection procedure. All cultures were shown to be free from mycoplasms.

Animals
NMRI nu/nu mice (B & M, Ry, Denmark) were used for xenografting at the age of 7-8 weeks (body weight 25-30 g males, 20-25 g females). The mice are homozygous for a recessive mutant gene designated nu and born athymic and thus lack detectable CD4+, CD8+ T-cell-activity. They can however develop cellular immunity with maturation. We chose to use young animals (< 3 months) to prevent this from happening and to be able to study the effects of treatment on growing animals. The mice were housed in an isolated room at 24 °C with a 12-h light, 12-h dark cycle. They were fed ad libitum with water and food pellets. The animal weight and general appearance were recorded every other day throughout the experiments. The experiments were approved by the regional ethics committee for animal research.

Xenografting
Tumor cells (30 x 10⁶ cells in 0.15 ml) were implanted s.c. in the hindleg of the animal, using a 23 G needle (Figure 5). Animals were anesthetized with 2 % Fluthane (Zeneca Ltd, Macclesfield, UK) supplemented with 50 % N₂O in oxygen. Tumor volume measurements began when the tumor became palpable (~0.1 ml) and were repeated every second day using a caliper. The tumor volume was calculated by the formula; 0.44 x length x width² (Wassberg et al. 1999). When the tumors reached a volume of ~300 mm³, the treatment began, after randomization of the animals. No randomized animal was excluded with the exclusion criteria applied, namely tumor rupture and body weight loss of > 10 %.
Administration of drugs
All animals received treatment within 2 to 4 weeks (SH-SY5Y and SK-N-AS) and within 7-10 weeks (IMR-32) after xenotransplantation. Control animals were given vehicle only. SU5416 was given at a dose of 50 mg/kg every other day (e.g. 175 mg/kg/week) or 100 mg/kg three times per week (e.g. 300 mg/kg/week) (paper I) s.c. in the neck. SU11657 was given orally daily (40 mg/kg/day), using a 1.2 mm Argyle umbilical vessel catheter (Sherwood Medical, St Louis, MO) (paper II). CHS 828 at a dose of 20 mg/kg was also given daily by oral gavage (paper III). TNP-470 was administered at 15 mg/kg every day or at 30 mg/kg every second day (papers III and IV) s.c. in the neck. Zoledronic acid was given at a dose of 100µg/kg every day s.c (paper IV).

Perfusion fixation and autopsy
The animals were anesthetized by an i.p. injection of 25 mg/kg of 2.2.2-tribromoethanol (Sigma) in 2.5 % 2-methyl-2-butanol (Sigma) in saline. A cannula was inserted in the thoracic aorta, and the animal was perfusion-fixed with 4 % paraformaldehyde in 1.47 mg/ml NaH2PO4 x H2O, 12.62 mg/ml Na2HPO4 x 2 H2O and 4.09 mg/ml in distilled H2O (Millonig’s
buffer, pH 7.4, 37 °C). The intra-arterial perfusion pressure was kept below 100 mm Hg. The thoracic and abdominal viscera were examined for macroscopic metastases. The true tumor weight and volume were recorded at autopsy and correlated well to the calculated volume.

Blood analyses
Blood was drawn from the retro-orbital venous plexus by inserting a small heparinized microhematocrit tube (Microcaps®, Drummond Scientific Company, Broomall, PA) behind the medial canthus of the eye. Blood was also drawn from the right ventricle, before perfusion fixation at killing, either with citrate plasma, 0.3 ml of 0.129 M sodium citrate, for a 2-3 ml blood sample (paper I) or with a heparinized syringe (papers II-IV). The blood was put on ice and spun within 20 min at 3,300 x g for 15 min. The plasma was removed and stored at –20 °C. Human VEGF-A165 (DVE00), PDGF-BB (DBB00) and SCF (DCK00) concentrations were measured with a specific sandwich ELISA according to the manufacturer’s instructions (Quantikine, R&D systems, Minneapolis, MN, USA). To ensure detection of intact VEGF-A, western blots from sera were performed using the same antibody as in the ELISA kit, and should show a distinct band at 23 kD (data not shown). Determinations of CHS 828 concentrations were performed by Leo Pharmaceuticals by use of an HPLC method with UV detection at 277 nm (Hjarnaa et al. 1997).

Tissue analyses
After perfusion fixation, the animals were examined for metastases and macroscopic organ changes. The tumors were dissected out and then immersion-fixed in 4% formaldehyde for approximately one week before dehydration and paraffin embedding. Sections were cut at 3 µm and put on 3-aminopropyltriethoxy-silane-treated glass slides (Sigma), dewaxed, rehydrated, and stained immunohistochemically.

Immunohistochemistry
To quantify tumor cell proliferation, staining for the Ki67 nuclear antigen was performed, which recognizes all cell cycle phases except G0 (Table 6, Figure 6 a). Staining specific for neuroendocrine and adrenergic cells, i.e., neuroblastoma cells, was performed by chromogranin A (CgA) and tyrosine hydroxylase (TH) immunohistochemistry (Table 6, Figure 6 b). Apoptosis were determined by the TdT-mediated dUTP-biotin nick end labelling (TUNEL).
The Apoptag® kit or the In situ Cell Death detection kit (Roche Diagnostics, Ingelheim, Germany) was applied (Table 6). For detection of VEGF-A (Figure 7a), VEGFR-2 (Figure 7b), PDGFR-β, and c-KIT see Table 7.

**Table 6. Overview of immunohistochemistry for Ki67, CgA, TH, and TUNEL used in paper I-IV** (ab= antibody)

<table>
<thead>
<tr>
<th>Substrate blocking</th>
<th>Ki67</th>
<th>CgA</th>
<th>TH</th>
<th>TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3% H₂O₂</td>
<td>0.3% H₂O₂</td>
<td>0.3% H₂O₂</td>
<td>0.3% H₂O₂</td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>20 min</td>
<td>20 min</td>
<td>20 min</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microwave treatment</th>
<th>Boric acid pH 7.0 or</th>
<th>Boric acid pH 7.0 or</th>
<th>Boric acid pH 7.0 or</th>
<th>Boric acid pH 7.0 or</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate buffer pH 6.0 (2 x 3 min, 750W)</td>
<td>Citrate buffer pH 6.0 (2 x 3 min, 750W)</td>
<td>Citrate buffer pH 6.0 (2 x 3 min, 750W)</td>
<td>Citrate buffer pH 6.0 (2 x 3 min, 750W)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blocking</th>
<th>1% BSA in PBS, 10 min at RT</th>
<th>1% BSA, 10% NRS in PBS, 20 min at RT</th>
<th>1% BSA in PBS, 20 min at RT</th>
<th>0.1% BSA in PBS, 10 min at RT</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Primary ab</th>
<th>Monoclonal mouse anti-Ki67 (MIB1, Dianova) 1:100, 1 h at RT</th>
<th>Monoclonal mouse anti-CgA (No. 1199021, Boheringer) 1:200, 30 min at RT</th>
<th>Monoclonal mouse anti-TH (No. 1017381, Boheringer) 1:500, 30 min at RT</th>
<th>TUNEL mix, 1 h at 37 °C, according to kit instructions</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Secondary ab</th>
<th>Polyclonal biotinylated rabbit anti-mouse Ig (E354, Dako) 1:200, 30 min at RT</th>
<th>Polyclonal biotinylated rabbit anti-mouse Ig (E354, Dako) 1:200, 30 min at RT</th>
<th>Polyclonal biotinylated rabbit anti-mouse Ig (E354, Dako) 1:200, 30 min at RT</th>
<th>Concenter POD, 30 min at 37 °C, according to kit instructions</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Positive control</th>
<th>Human breast carcinoma</th>
<th>Human jejunum</th>
<th>Human adrenal medulla</th>
<th>DNAse I 1 mg/ml, 10 min at RT</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Negative Control</th>
<th>Omission of primary ab</th>
<th>Omission of primary ab</th>
<th>Omission of primary ab</th>
<th>Replacement of TdT with water</th>
</tr>
</thead>
</table>

20
**Fig 6 a.** Ki67 expression in an SH-SY5Y control tumor at day 18 of therapy. Cell proliferate close to the central vessel. x 200.

**Fig 6 b.** TH expression in an IMR-32 SU11657 tumor at day 20 of therapy. x 200.

**Table 7.** Overview of immunohistochemistry for VEGF-A, VEGFR-2, PDGFR-β, and c-KIT used in paper I-IV.

<table>
<thead>
<tr>
<th>Substrate blocking</th>
<th>VEGF-A</th>
<th>VEGFR-2</th>
<th>PDGFR-β</th>
<th>c-KIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3% H2O2 0.3% H2O2 0.3% H2O2 0.3% H2O2</td>
<td>30 min 20 min 30 min 30 min</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microwave treatment</th>
<th>VEGF-A</th>
<th>VEGFR-2</th>
<th>PDGFR-β</th>
<th>c-KIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate buffer pH 760 (2x 3 min, 750W) Citrate buffer pH 6.0 (2x 3 min, 750W) Citrate buffer pH 6.0 (2x 3 min, 750W) Citrate buffer pH 6.0 (2x 3 min, 750W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 min, 750W</td>
<td>3 min, 750W</td>
<td>3 min, 750W</td>
<td>3 min, 750W</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blocking</th>
<th>VEGF-A</th>
<th>VEGFR-2</th>
<th>PDGFR-β</th>
<th>c-KIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% NRS in 0.1% BSA in PBS, 1 h at RT</td>
<td>1.5% NRS in PBS, 1 h at RT</td>
<td>1.5% NRS in PBS, 1 h at RT</td>
<td>1.5% NRS in PBS, 1 h at RT</td>
<td></td>
</tr>
<tr>
<td>30 min at RT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primary ab</th>
<th>VEGF-A</th>
<th>VEGFR-2</th>
<th>PDGFR-β</th>
<th>c-KIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal goat anti-human VEGF (R&amp;D, AB-293-NA)</td>
<td>Monoclonal mouse anti-Fk-1 (Santa Cruz, A3: Sc-6251)</td>
<td>Monoclonal mouse anti-PDGFR-β (Santa Cruz, A3: Sc-6252)</td>
<td>Polyclonal goat anti-c-KIT (Santa Cruz, A3: Sc-168-G)</td>
<td></td>
</tr>
<tr>
<td>1:50 over night in 4 °C</td>
<td>1:40 30 min at RT</td>
<td>1:40 30 min at RT</td>
<td>1:40 30 min at RT</td>
<td></td>
</tr>
</tbody>
</table>

21
<table>
<thead>
<tr>
<th>Secondary ab</th>
<th>VEGF-A</th>
<th>VEGFR-2</th>
<th>PDGFR-β</th>
<th>c-KIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal biotinylated rabbit anti-goat Ig (E0466, Dako), 1:200 30 min at RT</td>
<td>Polyclonal biotinylated rabbit anti-mouse Ig (E354, Dako)</td>
<td>Polyclonal biotinylated rabbit anti-mouse Ig (E354, Dako)</td>
<td>Polyclonal biotinylated rabbit anti-goat Ig (E0466, Dako)</td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>Fetal kidney</td>
<td>Human colon carcinoma</td>
<td>Human breast carcinoma</td>
<td>Human testicular germ cell tumor</td>
</tr>
<tr>
<td>Negative control</td>
<td>Omission of primary ab</td>
<td>Omission of primary ab</td>
<td>Omission of primary ab</td>
<td>Omission of primary ab</td>
</tr>
</tbody>
</table>

**Figure 7a.** VEGF-A expression in the tumor cell compartment in an SH-SY5Y control tumor at day 18. x 200

**Figure 7b.** VEGFR-2 expression in the tumor and endothelial cell compartments in an SK-N-AS control tumor at day 10. x 200.

All glasses were dewaxed and rehydrated and washed 3 x 5 min in PBS between every step except for after blocking in BSA or NRS. For detection, ABC/HRP was used followed by development with DAB, counterstaining with Harris’ hematoxylin for 20-30 s, and then mounted with Kaiser’s glycerol gelatine. All antibodies were diluted in 0.1 % BSA in PBS except for VEGFR-2, PDGFR-β, and c-KIT, which were diluted in 1.5% NRS in PBS.
To quantify angiogenesis, we used Bandeirea simplicifolia-1 lectin histochemistry for highlighting endothelial cells (Figure 8 b). After dewaxing, rehydration and blocking in H₂O₂ for 20 min, neuraminidase solution, N-2133 (Sigma) was applied on slides overnight at 37 °C (approximately 16 h). The biotinylated lectin, L3759 (Sigma), was applied at 1:100 and incubated at RT for 1 h. The sections were developed, counterstained, and mounted as above. As a positive control we used bovine capillary endothelial cells and omission of the neuraminidase solution served as a negative control. We compared stereological quantification of vascular parameters in 50 tumor sections, either solely by vascular perfusion fixation and counterstaining with hematoxylin and eosin (H&E) (where perfused blood vessels appear as punched-out holes in sections; Wassberg et al. 1999) (Figure 8 a), or by an identical preparation plus BS-1 lectin histochemistry to highlight endothelial cells. The two approaches yield identical results, but BS-1 highlighting made the counting procedure easier.

**Figure 8 a.** Hematoxylin & eosin stained vessels in an SK-N-AS control tumor after 10 days of therapy. Perfused blood vessels appear as punched-out holes in sections. x 200.

**Figure 8 b.** BS-1 stained vessels in an SK-N-AS control tumor after 10 days of therapy. Highlighting the vessels easier. x 200.

In-situ hybridization

Tissue sections on glass slides were dewaxed and rehydrated. After incubation in 0.2 M HCl the sections were permeabilized in proteinase K in 0.2 M Tris-HCl (pH 7.2), 2mM CaCl₂ followed by acetylation in triethanolamine (pH 8.0), 0.25% acetic anhydride. After rehydration and prehybridization for 2-4 h at 52-56°C, hybridization (2-6x10⁴ cpm/µl
hybridization solution) was carried out overnight in a humidified chamber containing 50% formamide/50% H₂O. The slides were washed in several steps and subsequently hybridization was performed in 10mM dithiothreitol (DTT) at hybridization temperature for 30 min and RNase A 100 µg/ml, followed by two treatment with sodium chloride-sodium citrate buffer (SSC) treatment for 30 min each to digest all unhybridized probe. After 24 h of autoradiography, NTB2 photographic emulsion (Eastman Kodak Co, Rochester, NY) diluted 1:1 with 2% glycerol in H₂O was applied and the slides were then exposed for 2-3 weeks. The slides were then developed and counterstained. The template used for hybridization was a 740-bp BamH1, HindIII VEGF-A₁₆₅ cDNA fragment (kindly provided by Dr. Arne Östman, Ludwig Institute for Cancer Research, Uppsala, Sweden). β-actin was used as a positive control and hybridization with the corresponding sense probe was used as a negative control.

Stereological quantification

A representative section from the geometrical center of each tumor was used. Structures were counted at x 400 with an eyepiece grid (506800, Leica, Singapore) with 10 x 10 squares (0.25 x 0.25 mm). The grid was placed at random at the upper left-hand corner of the section and systematically advanced every 1 to 3 mm, depending on the tumor size, in both directions by use of the goniometer stage of the microscope. Vascular parameters from 25 to 35 grids were quantified from each tumor (Wassberg et al. 1999) (Table 8).

In trained hands quantification of vascular parameters from one tumor section takes about 15-20 min. To adjust for the presence of apoptotic and necrotic areas, the presence of viable tissue in the uppermost square to the far right of the grid was noted (nvc, number of grids with viable corner) and was used as a correction factor in calculating vascular parameters. The nvc value was also used as an unbiased estimator of the fraction of viable tumor tissue. For quantification of the fraction of neuroblastoma cells staining positive for proliferation, apoptosis, TH, VEGF-A₁₆₅, and VEGF-A₁₆₅ mRNA a minimum of 2,000 cells per section were counted.
Table 8. Definition of vascular parameters

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Dimension</th>
<th>Equation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_v(\text{ves})$</td>
<td>0.0625 mm$^{-2}$</td>
<td>$L_v(\text{ves}) = 2 \times \Sigma Q_{\text{ves}}$ $/n_{vc} \times A_{\text{frame}}$</td>
<td>Mean number of vessels per counting frame</td>
</tr>
<tr>
<td>$L_v(\text{ves})$</td>
<td>mm$^{-2}$</td>
<td>$V_v(\text{ves}) = \Sigma P_{\text{ves}}$ $/n_{vc} \times P_{\text{pcg}}$</td>
<td>Length of vessels per tumor volume (length density)</td>
</tr>
<tr>
<td>$V_v(\text{ves})$</td>
<td>1</td>
<td>$V_v(\text{ves}) = \Sigma P_{\text{ves}}$ $/n_{vc} \times P_{\text{pcg}}$</td>
<td>Volume of vessels per tumor volume (volumetric density)</td>
</tr>
<tr>
<td>$S_v(\text{ves})$</td>
<td>mm$^{-1}$</td>
<td>$S_v(\text{ves}) = 2 \times \Sigma l_{\text{ves}}$ $/n_{vc} \times L_{\text{lig}}$</td>
<td>Surface area per tumor volume (surface density)</td>
</tr>
<tr>
<td>$a_v(\text{ves})$</td>
<td>mm$^2$</td>
<td>$a_v(\text{ves}) = V_v(\text{ves})$ $/L_v(\text{ves})$</td>
<td>Mean section area of vessels</td>
</tr>
<tr>
<td>$b_v(\text{ves})$</td>
<td>mm</td>
<td>$b_v(\text{ves}) = S_v(\text{ves})$ $/L_v(\text{ves})$</td>
<td>Mean boundary length of vessels</td>
</tr>
<tr>
<td>$d_v(\text{ves})$</td>
<td>mm</td>
<td>$d_v(\text{ves}) = 2 \times \sqrt{a_v(\text{ves})}$ $/n$</td>
<td>Mean section diameter of vessels</td>
</tr>
</tbody>
</table>

The eyepiece grid was used as an unbiased counting frame, a point-counting grid and a line-intercept grid. $Q_{\text{ves}}$, number of vessels in one counting frame; $n_{vc}$, number of grids with viable tissue in the uppermost square to the far right; $A_{\text{frame}}$, area of one counting frame; $P_{\text{ves}}$, number of test points hitting vessels (any layer or lumen) in one point-counting grid; $P_{\text{pcg}}$, number of test points in one point-counting grid; $l_{\text{ves}}$, number of intersections with vessels in one line intercept grid; $L_{\text{lig}}$, length of test lines in one line-intercept grid.

Statistical methods

Data were processed in Statistica 5.0 for Windows (StatSoft Inc., Tulsa, OK). Differences between two groups were analyzed with the Mann-Whitney U test. Differences where $p<0.05$ were considered statistically significant. When multiple comparisons between multiple treatments were necessary (paper III), the Kruskal-Wallis test was applied.
Results and discussion

Importance of Vascular Endothelial Growth Factor A in the Progression of Experimental Neuroblastoma (Paper I)
U Bäckman, Å Svensson, and R Christofferson

Disruption of the VEGF/VEGFR pathway represents a new attractive target for tumor therapy. SU5416 (SUGEN Inc.) is a small lipophilic, highly protein-bound, synthetic molecule which is mainly metabolized via the cytochrome P-450 enzymes (Mendel et al. 2000). SU5416 inhibits VEGFR-1 and -2 by blocking the intracellular signaling pathway (Itokawa et al. 2002, Mendel et al. 2000). In preclinical studies, SU5416 has been found to be a potent inhibitor of VEGF-mediated VEGFR-2 signaling (Fong et al. 1999, Shaheen et al. 1999, Ellis et al. 2000, Mendel et al. 2000). By its mechanism of action, blocking of angiogenesis through inhibition of VEGF signaling, SU5416 inhibits tumor growth in vivo but has little effect on tumor cells in vitro (Fong et al. 1999). SU5416 exerts a strong, rapid and long-lasting anti-proliferative effect on endothelial cells and inhibits the growth of multiple tumor types of various tissue origins in vivo (Fong et al. 1999, Sun et al. 1999).

We found that our SH-SY5Y neuroblastoma xenograft model expressed VEGF-A on both the mRNA and protein level and that expression of VEGF-A occurred mainly in the periphery of the perivascular cuffs of tumor cells. Treatment with SU5416 reduced the growth of neuroblastoma tumors by 65% with a treated/control (T/C) quotient of 0.35 for both dosage groups, i.e., 175 mg/kg/week and 300 mg/kg/week, and suppressed angiogenesis by approximately 40%. The reason for these results could be increased necrosis in the tumor i.e., that the tumor volume did not only represent viable tumor cells. No toxicity was seen. Kasahara et al. (2000) reported that in rats, chronic blockade of VEGF receptors by SU5416 induced apoptosis in type II alveolar cells and hence emphysema. But emphysema was not observed in our murine model.
SU5416 increased the fraction of apoptotic cells, and also caused upregulation of VEGF-A protein and mRNA expression. Reduction of angiogenesis could lead to increased hypoxia in the tumor and hence to increased apoptosis. At the same time, hypoxia can also induce expression of VEGF, which could explain the increased expression during treatment with SU5416. The plasma concentration of VEGF-A was significantly elevated in nude mice with tumors larger than 1.4 ml, and there was a moderate correlation between VEGF-A level in plasma and neuroblastoma tumor volume. Treatment with SU5416 led to a paradoxical increase in the VEGF-A concentration level per ml tumor volume during therapy. Thus, even though SU5416 prevents phosphorylation of VEGFR it seems that the VEGF-A production is constantly switched on and increased. This may be a direct intrinsic effect of SU5416 on neuroblastoma cells, or it could be due to increased hypoxia. Alternatively it could be a secondary effect of the “agonal differentiation” seen in the perivascular cuffs (Wassberg et al. 1999), where excessive VEGF can be released from agonally differentiated and dying tumor cells.

SU5416 is currently being tested in several ongoing clinical trials both as single treatment and in combination with cytotoxic substances. SU5416 is in phase III for metastatic colorectal cancer (de Primo et al. 2003). The maximum tolerated dose for i.v. administration of SU5416 is 145 mg/m² given twice weekly (Rosen et al. 1999, Cropp et al. 1999). Dose-limiting toxicities consisted of projectile vomiting, nausea, and severe headache. Other adverse events are superficial and deep vein phlebitis, diarrhea, and fatigue. No hematological or organ toxicities have been seen. Unfortunately, SU5416 has not been found to be as efficient in human tumors as in animal models (Kuenen et al. 2003). Perhaps patients with large tumors have developed a population of vessels, instead of immature VEGF-dependent vessels, or the bioavailability of SU5416 may be insufficient because of its relatively short plasma half-life (12 h). Yet another explanation could be that colorectal tumor cells produce other growth factors besides VEGF.

In conclusion, we suggest that the new drug SU5416 may be of therapeutic benefit in neuroblastoma patients, with limited side effects compared to chemotherapeutic drugs, but that the inhibition of one key receptor may not imply complete inhibition of angiogenesis.
The Selective Class III/V Receptor Tyrosine Kinase Inhibitor SU11657 Inhibits Tumor Growth and Angiogenesis in Experimental Neuroblastomas Grown in Mice (Paper II)

U Bäckman, and R Christofferson

Vascular endothelial growth factor, fibroblast growth factor, and platelet-derived growth factor and their cognate receptor tyrosine kinases are strongly implicated in angiogenesis associated with solid tumors. Apart from regulating angiogenesis, VEGF, PDGF, and stem cell factor can serve as autocrine growth and survival factors for neuroblastoma cells (Pietras et al. 2001). Further, PDGF upregulates VEGF and stimulates recruitment of pericytes and fibroblast-like cells required for stabilization of capillaries during angiogenesis (Shawver et al. 2002). SU11657 (SUGEN, Inc) (Pollard et al. 2002, Sohal et al. 2003) is a selective multi-targeted tyrosine kinase inhibitor with anti-tumor and anti-angiogenic activity exerted through targeting of PDGF receptors, VEGF receptors, c-KIT and FLT3. In the present study, oral treatment with SU11657 reduced the tumor growth rate by 90% (SK-N-AS), 88% (SH-SY5Y), and 93.8% (IMR-32).

When the treatment with SU11657 was continued for 40 days, the tumors kept on growing, but slowly. At day 40 the tumor volume had increased by 24 % (SK-N-AS), 4.6 % (SH-SY5Y), and 83 % (IMR-32) compared to days 10, 18 and 20, respectively. The treated animals did not show any signs of toxicity. The fraction of apoptotic tumor cells was significantly increased by therapy at days 10, 18, and 20. Apoptosis was even more increased at day 40, while proliferation was unaffected. These results were predicted since SU11657 treatment blocks several survival signals received by vessels via the receptor tyrosine kinases, resulting in endothelial death by apoptosis and vascular regression, which in turn leads to tumor cell stress and subsequent tumor cell apoptosis. Treatment with SU11657 reduced the plasma concentration of VEGF-A in all groups (Figure 8 a). In contrast, there was an increase in the PDGF-BB and SCF concentrations during therapy (Figure 8 c and e). It is possible that the efficacy of SU11657 is different for different receptors and that the blocking of VEGFR was more effective, or that other factors act as survival factors, e.g., PDGF-BB and SCF. Interestingly, MYCN-amplified IMR-32 exhibited low circulating concentrations of VEGF-A, but significantly higher concentrations of SCF. Also, IMR-32 was the cell line which exhibited the lowest increase in apoptosis in response to therapy.
Figure 8. Plasma levels of a) VEGF-A, b) VEGF-A/ml tumor volume, c) PDGF-BB, d) PDGF-BB/ml tumor volume, e) SCF, and f) SCF/ml tumor volume in the neuroblastoma cell lines SK-N-AS, SH-SY5Y, and IMR-32. ■—Controls, ●—SU11657 treatment for 10, 18 and 20 days respectively, ■—SU11657 treatment for 40 days
This might be explained by the observation that SCF is produced by neuroblastoma cells and protects them from apoptosis via an autocrine loop. However, when considering the plasma concentrations per milliliter tumor volume, we found an upregulation of VEGF-A, PDGF-BB and SCF in plasma at days 10, 18, and 20 of SU11657 therapy (Figure 8 b, d, and f). This phenomenon could be due to release of growth factors from apoptotic and necrotic cells, i.e. dying tumor cells, or to increased hypoxia. We ruled out the possibility that it might be an intrinsic effect of SU11657, since animals with no tumor that only received SU11657 had very low levels of the growth factors. At day 40 the plasma concentrations of all three growth factors per milliliter tumor volume had decreased compared to those on days 10, 18, and 20 for all three cell lines (Figure 6). This could indicate either that the tumors had elaborated other pathways to attract new vessels, or that the growth factors liberated to the circulation due to necrosis had been cleared from the circulation, or that the functional tumor burden had decreased. The VEGF-A immunohistochemistry followed the same pattern as the plasma concentrations, with a flare during short-term therapy and reduced levels after 40 days of therapy. The latter could be due to a decreased number of viable tumor cells after long-term therapy.

It has been shown previously that SH-SY5Y expresses both c-KIT and VEGFR-2 (Cohen et al. 1994, Meister et al. 1999), but we have demonstrated for the first time by immunohistochemistry that the three neuroblastoma cell lines also expressed the VEGFR-2, PDGFR-β and c-KIT receptors. VEGFR-2 and PDGFR-β were expressed in tumor cell and endothelial cell compartments and in areas of apoptosis and necrosis. C-KIT was also expressed in tumor cell and endothelial cell compartments but not in apoptotic or necrotic areas. C-KIT expression was less frequent in SH-SY5Y, where only 40 % of the untreated tumors expressed the receptor. The expression of the receptors decreased during therapy and after 40 days of treatment only a few vessels stained positively for the receptors (VEGFR-2, PDGFR-β, and c-KIT). These changes could be due to suppressed angiogenesis in the tumors.

New compounds that target several different growth factors, such as SU11657, SU6668 and SU11248, and/or combinations of new biological compounds that target different pathways involved in angiogenesis, such as integrins, matrix metalloproteinases, and cell adhesion molecules have to be explored. SU11657 is now entering phase I clinical trial (Sohal et al. 2003, Pollard et al. 2003) and is showing promising results. In conclusion, this new compound was more potent than SU5416, as a single agent in our xenograft model, without causing any toxicity. Combining these results we suggest that SU11657 may be a candidate for neuroblastoma therapy in children.
CHS 828 Inhibits Neuroblastoma Growth in Vivo Alone and in Combination with Antiangiogenic Drugs (Paper III).

Å Svensson, U Bäckman, E Jonsson, R Larsson, and R Christofferson

CHS 828 belongs to a new class of chemotherapeutic drugs, the pyridyl cyanoguanidines. It has potent antitumor activity both in vitro and in vivo. It shows low correlation with the activity patterns of known cancer drugs, and of known patterns of multidrug resistance (Hjarnaa et al. 1999). CHS 828 has structural similarities with the catecholamine precursor metaiodobenzylguanidine (MIBG), which is known to accumulate in neuroblastomas and is used clinically for imaging and radiotherapy (Montaldo et al. 1991, Troncone et al. 1997). Clinical trials with angiogenesis inhibitors as adjuvants to chemotherapeutic drugs are currently in progress (Deplanque and Harris 2000). In preclinical tests, combination therapy has proven successful in treatment of multi-drug resistant tumors, and has enhanced the efficacy of the chemotherapeutic drug alone (Kakeji and Teicher 1997, Browder et al. 2000, Klement et al. 2000).

We found that CHS 828, administered daily, reduced the growth of SH-SY5Y human neuroblastoma tumors in NMRI nu/nu mice by 82 % without apparent toxicity. CHS 828 resulted in a total tumor regression in 4/9 animals. Combination therapy with CHS 828 and the antiangiogenic drugs TNP-470 and SU5416 decreased neuroblastoma growth by a further 10 and 3 %, respectively. The dramatic effect of CHS 828 made analysis of possible potentiation by angiogenic inhibition difficult. In a separate study we treated animals with only CHS 828 for just 4 days, but already at that time the tumors were so necrotic that it was impossible to analyze the occurrence of proliferation, apoptosis, and angiogenesis in the tumor sections.

CHS 828 has been in phase I clinical trials in patients with solid tumor malignancies (Hovstadius et al. 2002). CHS 828 was administrated to fasting patients as a single oral dose on days one to five of each treatment cycle, and the patients received one to six cycles of treatment. The doses ranged from 30 mg to 200 mg as a total dose in the cycle (plasma half-life 2.1 h). Hematological toxicity was generally mild and dominated by transient thrombocytopenia and lymphocytopenia. Other toxic effects were nausea, vomiting, diarrhea, fatigue, and localized genital mucositis. No objective tumor responses were observed, and the patients showed stable disease after two courses of therapy. CHS 828 is on its way in to phase II clinical trials, at doses of 20 mg once daily for five days in cycles of 28 days’ duration. TNP-470 is in phase III clinical trials and is given in combination with several different chemotherapeutics.
Thus much is still unclear concerning dosages and optimal ways of administration of these new drugs alone or in combination with conventional chemotherapy. When applied clinically, caution must be observed with combinations of novel biological agents and chemotherapy. In conclusion, the new therapeutic drug CHS 828 alone, as well as in combination with angiogenic inhibitors, is a potent inhibitor of experimental neuroblastoma growth in vivo in doses not causing systemic toxicity. Our study and previous investigations of CHS 828 have shown low toxicity and no patterns of multi-drug resistance, which makes it promising as a new drug in the treatment of childhood cancers.

The Bisphosphonate Zoledronic acid Reduces Experimental Neuroblastoma Growth by Interfering with Tumor Angiogenesis
Ulrika Bäckman, Åsa Svensson and Rolf Christofferson

The bisphosphonates are a family of pyrophosphate analogues (Fleisch et al., 1998). These compounds have high affinity for bone mineral (Jung et al., 1973) and are potent inhibitors of osteoclastic bone resorption (Schenk et al. 1973). They are effective in the treatment of a variety of conditions associated with increased bone resorption, including malignant bone disease, Paget’s disease, and osteoporosis (Fleisch et al. 1996). However, high doses of some of the earlier bisphosphonates can paradoxically lead to impaired mineralization (Reitsma et al. 1980). Zoledronic acid is a new member of the bisphosphonate class of compounds. These compounds have reduced the growth of several cancer cell lines in vitro, and have recently been shown to be inhibitors of angiogenesis (Green, 2000). However, zoledronic acid is not yet approved for use in children. The angiogenic inhibitor TNP-470 (Ingber et al. 1990), a synthetic analogue of the fungal antibiotic fumagillin, has been shown to inhibit the growth of multiple tumors in vivo.

In this study we have compared the effects of zoledronic acid with those of TNP-470 in a nude mouse xenograft model for the childhood cancer neuroblastoma. We show here for the first time that zoledronic acid, initially developed as an inhibitor of bone resorption, potently reduces the growth rate of neuroblastoma in a nude mouse xenograft model. Zoledronic acid reduced the tumor growth rate by 33 % (T/C=0.67) without apparent toxicity. We speculated that this tumor growth reduction could have been more pronounced if zoledronic acid had not been cleared from the circulation by binding to bone mineral. We investigated the anti-proliferative effect of steady state sera from zoledronic acid - or TNP-470 - treated animals on bovine capillary endothelial cells in vitro. Moderate inhibition
was observed on treatment with zoledronic acid (p=0.14) and TNP-470 (15 mg/kg/day and 30 mg/kg/eod) (p=0.12), compared to sera from controls animals. These data indicate that even if the plasma half-life is short, some interference with tumor angiogenesis can be identified.

Tumors in zoledronic acid-treated animals exhibited a tendency to a reduction in vessel length and area, while the vessel volume was paradoxically increased by 22.5%. TNP-470 was surprisingly less effective and reduced the tumor growth rate by a moderate 26% (15 mg/kg/day, mean volume of treated tumors/mean volume of control tumors, T/C = 0.74) and 11% (30 mg/kg every other day, T/C = 0.89) respectively. The difference in tumor growth between the two dosage groups, although not significant, indicates that the drug may be more effective upon frequent administration.

Zoledronic acid is currently in phase III clinical trials in patients with breast cancer, multiple myeloma, prostate cancer, lung cancer, and other solid tumor types. It has been shown that breast cancer patients treated with zoledronic acid have a 20% lower risk of developing bone metastases compared to patients treated with other bisphosphonates, e.g. pamidronate. The most common adverse effects of zoledronic acid are flu-like syndrome (fever, arthralgia, myalgia), skeletal pain, fatigue, weakness, and edema. In conclusion, we show here for the first time that the bisphosphonate zoledronic acid is a potent inhibitor of neuroblastoma growth, and that one mechanism of action may be interference with tumor angiogenesis. This new finding implies that bisphosphonates may have additional beneficial properties in patients with cancer apart from the inhibitory effect on osteoclastic bone resorption. Furthermore, this is the first report of anti-tumor activity of zoledronic acid on soft tissue tumors at non-bone sites.
Conclusions

- Our xenograft models are relevant, reliable and give reproducible results.
- SH-SY5Y neuroblastoma cells express the angiogenic stimulator VEGF-A on both the mRNA and protein levels.
- SH-SY5Y, IMR-32, and SK-N-AS neuroblastoma cell lines express VEGFR-2, PDGFR-β, and c-KIT receptors.
- The VEGFR signaling inhibitor, SU5416, reduced the tumor growth rate by 65% and tumor angiogenesis by 40% without toxicity. This suggests that the interference with VEGF-signalling may be beneficial in the treatment of neuroblastoma.
- SU11657 reduces the tumor growth rate (88-94 %) and angiogenesis (63-96 %), even in MYCN-amplified cell lines, without toxicity.
- The new cytotoxic substance CHS 828 reduced the tumor growth rate by 82 % and induced total tumor regression in 44 % of the animals, without toxicity.
- TNP-470 or SU5416, when used in combination with CHS 828, decreased neuroblastoma growth by a further 10 and 3 % respectively compared to CHS 828 alone.
- The bisphosphonate zoledronic acid is a potent inhibitor of neuroblastoma growth, reducing it by 33 %, and it may exert its action by interference with tumor angiogenesis. Futhermore, this is the first report of anti-tumor activity of zoledronic acid on soft tissue tumors at a non-bone site.
General discussion and future perspectives

Currently, a variety of chemotherapeutic drugs are being used to treat cancer. Unfortunately, some of them have hold limited efficacy, due to problems of delivery, penetration, and lack of selectivity for the tumor cells, thereby causing damage to healthy tissues. Another major clinical problem is the development of acquired drug resistance. Tumor cells represent a rapidly changing target on account of their genetic instability, heterogeneity, and high rate of mutation. In contrast, endothelial cells are not transformed, and they are bone marrow-derived – and the bone marrow never acquires resistance to chemotherapeutics.

Angiogenic inhibition, which targets activated endothelial cells, offers several advantages over therapy directed against tumor cells, but what about the future clinical values of angiogenic inhibitors in pediatric oncology? For neuroblastoma, the survival of patients with advanced disease is still poor and there is a clinical need for new treatment strategies, based, for instance, on induction of differentiation, apoptosis, or inhibition of tumor angiogenesis.

Several in vitro studies using endothelial cells, isolated from either capillaries or large vessels, have given an insight into the molecular and cellular biology of angiogenesis (Montesano et al. 1992). However, to evaluate the potency of angiogenic inhibitors it is important to have relevant animal models. An increasing number of angiogenic inhibitors have been identified, many of which have been shown to have anti-angiogenic activity in a particular assay, such as the avian chorio-allantoic membrane assay. More recently, researchers have focused on the search for compounds with a specific effect on critical step of the angiogenic process. This includes inhibitors of cell invasion, motility, and adhesion, inhibitors of activated endothelial cells, compounds that interfere with angiogenic growth factors or their receptors, or vascular targeting. Today, more than 40 angiogenic inhibitors have been characterized and some of these are in clinical trials against cancer (Lievens et al. 2001)

Angiostatic therapy in combination with other treatment modalities is another approach in the treatment of cancer. Some angiogenic inhibitors
have been shown to enhance the response of murine tumors to chemotherapy or radiotherapy. Even though the mechanism is not yet completely understood, these studies may represent what is becoming a paradigm shift in the type of adjuvant treatment to be used with traditional chemotherapy or radiotherapy (Kozin et al. 2001, Teicher et al. 1995, Gorski et al. 1994). In the future, use of new compounds that target several growth factor receptors may be a good strategy in attempts to inhibit angiogenesis in tumors. Unfortunately, clinical trials are designed for chemotherapeutics, with the maximum tolerated dose given for a maximum of 4 weeks followed by 4 weeks of recovery in patients with end-stage disease that has not responded to conventional therapy, i.e. with large, bulky, metastatic tumors, presumably with a considerable proportion of mature vessels. With angiogenic inhibitors, less toxicity is seen, and therefore the “optimal biological dose” seems to be more relevant than the “maximum tolerated dose” in therapy (Deplanque and Harris, 2000). Angiogenic inhibitors should be given continuously for a relatively long period without any break, as otherwise angiogenesis in the tumor may start again.

Finally, angiogenic inhibition may prove useful in combination with therapies aimed directly at tumor cells. As each therapy is aimed at a different cellular target, the hope is that in treating children with cancer combinations will prove to be more effective Thus, clinical trials are ongoing with new therapeutic strategies, including angiogenic inhibitors, but the current design of clinical trials is not optimal for angiogenic inhibition. It is my belief that angiogenic inhibitors are of clinical value in pediatric oncology. In view of their low toxicity, it seems rational to give angiogenic inhibitors continuously in a low dose after surgical resection, alone or in combination with chemotherapy.


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