

The Selective Class III/V Receptor Tyrosine Kinase Inhibitor SU11657 Inhibits Tumor Growth and Angiogenesis in Experimental Neuroblastomas Grown in Mice

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ABSTRACT

Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) and their cognate receptor tyrosine kinases are strongly implicated in angiogenesis associated with solid tumors. SU11657 (SUGEN) is a selective multitargeted tyrosine kinase inhibitor with antitumor and antiangiogenic activity exerted by targeting PDGF receptors (PDGFR), VEGF receptors (VEGFR), stem cell factor receptor (c-KIT), and FMS-related tyrosine kinase 3. Oral administration of SU11657 at $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ to athymic mice resulted in significant growth inhibition of a panel of s.c. human neuroblastoma xenografts, namely, fast-growing SK-N-AS, MYCN-amplified IMR-32, and SH-SY5Y, by 90, 93.8, and 88%, respectively, and was well tolerated. All of the cell lines expressed VEGFR-2, PDGFR- β , and c-KIT protein in the tumor cell and endothelial cell compartment by immunohistochemistry, and the expression decreased during therapy. Plasma concentrations of VEGF-A, PDGF-BB, and stem cell factor increased per milliliter of tumor volume at days 10, 18, and 20 of therapy. Furthermore, SU11657 reduced tumor angiogenesis by 63–96%. Our experimental data suggest that the angiogenesis inhibitor SU11657 may be beneficial in the treatment of

rapidly growing and highly vascularized solid tumors of childhood, such as neuroblastoma. In summary, the class III/V receptor tyrosine kinases and their ligands are implicated in angiogenesis, tumor cell proliferation, and cell survival, and it seems reasonable to determine whether interference with these pathways can suppress neuroblastoma growth or not. (*Pediatr Res* 57: 690–695, 2005)

Abbreviations

CgA, chromogranin A
C-KIT, stem cell factor receptor
FL, flt-3 ligand
FLT3, fms-related tyrosine kinase 3
PDGF, platelet-derived growth factor
PDGFR- β , platelet-derived growth factor receptor beta
RTK, receptor tyrosine kinase
SCF, stem cell factor
TH, tyrosine hydroxylase
VEGF-A, vascular endothelial growth factor A
VEGFR-1/2, vascular endothelial growth factor receptor 1/2.

Neuroblastoma is one of the most common extracranial solid tumors in children (1,2). Its biologic behavior is intriguing; some tumors regress spontaneously, whereas others progress despite aggressive multimodal therapy. Patient age >1 y, advanced tumor stage, and amplification of the MYCN oncogene are three established negative prognostic factors (3). Improving the survival quality of rate and life for the survivors of childhood cancer underlines the importance of finding new treatment strategies.

Angiogenesis is essential for tumor growth and metastasis. Tumor angiogenesis is driven by angiogenic growth factors

secreted by tumor cells (4). Among these factors, vascular endothelial growth factor A (VEGF-A) is probably the most important for the development, differentiation, and maintenance of the tumor vascular system and is considered to play a key role in angiogenesis (4). VEGF interacts with cell surface receptors, in humans kinase domain-containing receptor or VEGF receptor-2 (VEGFR-2) and Fms-like tyrosine kinase (Flt-1) or VEGFR-1, which is expressed almost exclusively on vascular endothelial cells (5). Neuroblastoma cell lines as well as most primary tumors express the mitogenic VEGFR-2, and VEGFR-2 is involved in malignant transformation of neuroblastoma cells (6).

Apart from regulating angiogenesis, VEGF, platelet-derived growth factor (PDGF), and stem cell factor (SCF) and their receptors can also serve as autocrine growth and survival factors for neuroblastoma cells and have been implicated in progression of neuroblastoma (7). Neuroblastoma cells express

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PDGF A- and B-chains and PDGF receptor- α (PDGFR- α) and PDGFR- β . PDGF isoforms are involved in neuroblastoma cell growth, as well as neuronal cell migration, growth, and differentiation in human brain development (8). PDGF up-regulates VEGF and stimulates recruitment of pericytes and fibroblast-like cells that are required for stabilization of capillaries during angiogenesis (9). Another class III/V receptor tyrosine kinase (RTK) is c-KIT, which is essential for the development of hematopoietic cells, and it has been proposed that c-KIT may be up-regulated in acute myeloid leukemia (10). Cohen *et al.* (11) were the first to assume the function of the autocrine loop between the SCF and its receptor (c-KIT). A recent study indicated that the main role of SCF in neuroblastomas could be that of protecting cells from apoptosis (12). The 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 an accelerated angiogenesis (13). FLT3 and its ligand (FL) are expressed in neuroectodermal tumors and in neuroblastomas, where they promote the survival and proliferation of tumor cells. SCF and FL often show synergic or additive activity on the hematopoietic progenitors (14).

On the basis of the above, we set out to explore the activity of SU11657 (SUGEN, South San Francisco, CA), a small-molecule, synthetic, multitargeted inhibitor of class III/V RTKs, in preclinical models of neuroblastoma. We found that SU11657 exhibited potent antitumor and antiangiogenic activity in these models, indicating the potential importance of class III/V RTKs in neuroblastoma.

METHODS

SU11657. SU11657 (15) was a gift of SUGEN. SU11657 was suspended in 0.5% (wt/vol) carboxymethylcellulose sodium (medium grade), 0.9% (wt/vol) sodium chloride, 0.4% (wt/vol) polysorbate 80, and 0.9% (wt/vol) benzyl alcohol in deionized water to the appropriate concentration. SU11657 was administered orally once daily at a dose of 40 mg/kg.

Neuroblastoma cells. Three human neuroblastoma cell lines were used in this study: SH-SY5Y, SK-N-AS, and the MYCN-amplified IMR-32 was purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured as described previously (16). For SK-N-AS and IMR-32, 1% nonessential amino acids were added to the medium (Sigma Chemical Co., St. Louis, MO). All cells were shown to be free from mycoplasmas.

Animals. Ninety nude male NMRI nu-nu mice (B&M, Ry, Denmark) were used for xenografting at the age of 6–7 wk. The animals were housed in an isolated room at 23°C with a 12-h light/dark cycle. They were fed *ad libitum* with water and food pellets. The experiment was approved by the regional ethics committee for animal research, Uppsala, Sweden.

Xenografting. The xenografting and tumor measurements were performed as described previously (16,17). When the tumors had reached 0.3 mL, the animal was randomized into one of three groups: control animals that received oral vehicle only ($n = 10$ –11/cell line); animals that received SU11657 orally every day for up to 20 d, through a 1.2-mm Argyle umbilical vessel catheter (Sherwood Medical, St. Louis, MO; $n = 10$ /cell line); or animals that were given long-term treatment (40 d) with SU11657 ($n = 9$ /cell line). The animals were treated for 10 (SK-N-AS), 18 (SH-SY5Y), or 20 (IMR-32) d. When the tumor burden in control animals approached a maximum volume of 4–5 mL, the animals were anesthetized and perfusion-fixed as previously described (16).

Blood analyses. Blood was drawn from the retro-orbital venous plexus by inserting a small heparinized microhematocrit tube behind the medial canthus of the eye at day 5 (SK-N-AS), 9 (SH-SY5Y), or 10 (IMR-32). Blood was also drawn from the right ventricle with a heparinized syringe before perfusion fixation at killing. The heparinized blood was put on ice and spun within 20 min at $3300 \times g$ for 15 min. The plasma was removed and stored at -20°C .

ELISA. Human VEGF (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).

Tissue analyses. To quantify tumor cell proliferation, we performed staining for the Ki67 nuclear antigen. Staining specific for neuroendocrine and adrenergic cells was performed by chromogranin A (CgA) and tyrosine hydroxylase (TH) immunohistochemistry. Apoptosis was determined by the TdT-mediated dUTP-biotin nick end labeling method (16). For detection of VEGF-A (16) and for VEGFR-2, PDGFR- β , c-KIT, and quantification of angiogenesis (BS-1) see Table 1.

All glasses were dewaxed, rehydrated, and washed three times for 5 min in PBS between every step. For detection, ABC/horseradish peroxidase was used followed by development with diaminobenzidine, counterstained with Harris' hematoxylin for 20 s, and mounted with Kaiser's glycerol gelatin. 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% rabbit serum in PBS.

Stereologic quantifications. Stereologic quantification was performed as previously described (17).

RESULTS

Neuroblastoma growth. Oral treatment with SU11657 potently suppressed neuroblastoma growth from all cell lines (SK-N-AS, SH-SY5Y, and IMR-32; Fig. 1). The treated/control quotient for SK-N-AS after 10 d of therapy was 0.10 ($p < 0.001$; Fig. 1A), for SH-SY5Y after 18 d of therapy 0.11 ($p < 0.001$; Fig. 1B), and for IMR-32 after 20 d of therapy 0.06 ($p < 0.001$; Fig. 1C). When the treatment was continued for 40 d ($n = 9$ /cell line), the

Table 1. Overview of immunohistochemistry

	VEGFR-2	PDGFR- β	c-KIT	BS-1
Substrate blocking	0.3% H ₂ O ₂ , 30 min	0.3% H ₂ O ₂ , 30 min	0.3% H ₂ O ₂ , 30 min	0.3% H ₂ O ₂ , 30 min
Microwave treatment	Citrate buffer pH 6.0 (2 \times 3 min, 750 W)	Citrate buffer pH 6.0 (2 \times 3 min, 750 W)	Citrate buffer pH 6.0 (2 \times 3 min, 750 W)	
Blocking	1.5% NRS in 0.1% BSA, 30 min at room temperature	1.5% NRS in 0.1% BSA, 30 min at room temperature	1.5% NRS in 0.1% BSA, 30 min at room temperature	Neuroaminidase solution (N2133; Sigma) at 37°C overnight
Primary antibody	Monoclonal mouse anti-Fli1 (Santa Cruz; A3:Sc-6251), 1:40, 30 min at room temperature	Monoclonal mouse anti-PDGFR- β (Santa Cruz; A3: Sc-6252), 1:40, 30 min at room temperature	Monoclonal mouse anti-c-KIT (Santa Cruz, A3:Sc-168-G), 1:40, 30 min at room temperature	Biotinylated lectin (L3759; Sigma) 1:100, 1 h at room temperature
Secondary antibody	Polyclonal biotinylated rabbit anti-mouse Ig (E354, Dako) 1:200, 30 min at room temperature	Polyclonal biotinylated rabbit anti-mouse Ig (E354; Dako) 1:200, 30 min at room temperature	Polyclonal biotinylated rabbit anti-mouse Ig (E354; Dako) 1:200, 30 min at room temperature	
Positive control	Human colon carcinoma	Human breast carcinoma	Human testicular germ cell tumor	Bovine capillary endothelial cells
Negative control	Omission of primary antibody	Omission of primary antibody	Omission of primary antibody	Omission of neuroaminidase solution

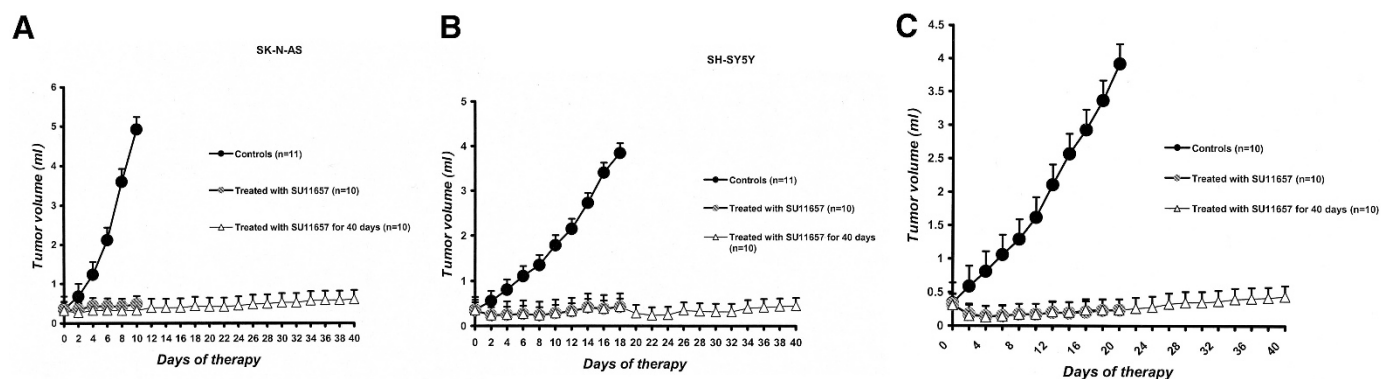


Figure 1. (A) Growth rate of xenotransplanted human SK-N-AS neuroblastoma cells. When the tumor volume was 0.3 mL, treatment began. ●, controls ($n = 11$); ●, SU11657, $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ($n = 10$) for 10 d; △, SU11657, $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ($n = 10$) for 40 d. $p < 0.001$ at day 2 of therapy. Mean tumor volume ± 1 SD. (B) Growth rate of xenotransplanted human SH-SY5Y neuroblastoma cells. When the tumor volume was 0.3 mL, treatment began. ●, controls ($n = 11$); ●, SU11657, $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ($n = 10$) for 18 d; △, SU11657, $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ($n = 10$) for 40 d. $p < 0.001$ at day 4 of therapy. Mean tumor volume ± 1 SD. (C) Growth rate of xenotransplanted human IMR-32 neuroblastoma cells. When the tumor volume was 0.3 mL, treatment began. ●, controls ($n = 10$); ●, SU11657 $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ($n = 10$) for 20 d; △, SU11657, $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ($n = 10$) for 40 d. $p < 0.001$ at day 4 of therapy. Mean tumor volume ± 1 SD.

tumors continued to grow in all groups but slowly (Fig. 1). No toxicity was seen. The weight gain in all treatment groups was similar to that in the corresponding control group. The SU11657-treated tumors were smaller, paler, and harder than the control tumors. No tumor regression was seen in either of the treatment groups. The reason for the different lengths of treatment was the finding of different growth rates of tumors in control animals.

Angiogenesis and viable tissue. The results of stereologic quantification of angiogenesis are presented in Table 2. At day 10, 18, or 20, there was a significant reduction of the number of vessels per grid, the length of vessels per tumor volume, the volume of vessels per tumor volume, and the surface area of vessels per tumor volume in all of the treatment groups compared with the controls. With all cell lines, the SU11657 treatment seemed to have decreased the viable tumor tissue fraction at 10, 18, and 20 d of therapy. After treatment for 40 d, the fraction of viable tissue tended to be further decreased, significantly in SK-N-AS and IMR-32.

Expression of VEGFR-2, PDGFR- β , and c-KIT. VEGFR-2 and PDGFR- β protein was detected in the tumor cell compartment and endothelial cell compartment, as well as in areas of apoptosis and necrosis, in all three cell lines. The expression decreased during therapy. C-KIT was expressed in the tumor cell and endothelial cell compartments in SK-N-AS and SH-SY5Y, but only SH-SY5Y expressed c-KIT in areas of apoptosis and necrosis. The MYCN-amplified IMR-32 did not show any c-KIT staining. The expression of VEGFR-2, PDGFR- β , and c-KIT in the endothelial cell compartment decreased during therapy with all three cell lines (data not shown).

Expression of VEGF protein. The expression of VEGF-A at the protein level was significantly increased during therapy at days 10 and 18 in SK-N-AS and SH-SY5Y but not in IMR-32. There was no significant difference between expression of VEGF-A at day 40 compared with days 10, 18, and 20 (Table 3).

Apoptosis and proliferation. The fraction of apoptotic neuroblastoma cells was significantly increased during therapy.

Table 2. Quantification of vascular parameters at days 10, 18, 20, and day 40 of therapy for SK-N-AS, SH-SY5Y, and IMR-32

Cell line	SK-N-AS			SH-SY5Y			IMR-32		
	Control ($n = 11/11$)	SU11657 (10 days) ($n = 10/10$)	SU11657 (40 days) ($n = 9/9$)	Control ($n = 11/11$)	SU11657 (18 days) ($n = 10/10$)	SU11657 (40 days) ($n =$ 9/9)	Control ($n = 11/11$)	SU11657 (20 days) ($n = 10/10$)	SU11657 (40 days) ($n = 9/9$)
Q_v	111.4 ± 31.8	13.3 ± 7.3	4.7 ± 2.4	75.6 ± 7.3	13.6 ± 5.3	8.2 ± 1.6	59.7 ± 33.4	6.25 ± 2.3	4.71 ± 1.6
	change (%)	-88.1‡	-64.7*†	change (%)	-82.0‡	-39.7*†	change (%)	-89.5‡	-24.6*†
L_v (mm^{-2})	190.1 ± 82.2	26.4 ± 14.8	12.3 ± 7.9	124.6 ± 46.6	24.9 ± 9.6	19.8 ± 5.2	83.6 ± 41.6	10.1 ± 6.7	8.9 ± 3.2
	change (%)	-86.1‡	-53.4*†	change (%)	-80.0‡	-20.5* ^{n.s.}	change (%)	-87.9‡	-11.9* ^{n.s.}
V_v (1)	0.51 ± 0.03	0.07 ± 0.05	0.07 ± 0.04	0.24 ± 0.08	0.08 ± 0.03	0.07 ± 0.05	0.46 ± 0.11	0.17 ± 0.05	0.17 ± 0.03
	change (%)	-86.3‡	0.0* ^{n.s.}	change (%)	-66.7‡	-12.5* ^{n.s.}	change (%)	-63.0‡	0.0* ^{n.s.}
S_v (mm^{-1})	10.7 ± 0.9	1.9 ± 0.9	1.9 ± 0.8	6.8 ± 0.9	1.6 ± 0.9	1.4 ± 0.5	9.9 ± 5.1	2.7 ± 1.9	2.6 ± 1.2
	change (%)	-82.2‡	0.0* ^{n.s.}	change (%)	-76.4‡	-12.5* ^{n.s.}	change (%)	-72.7‡	-3.7* ^{n.s.}
d (μm)	18.2 ± 3.8	20.6 ± 9.2	20.1 ± 5.5	18.5 ± 4.5	18.9 ± 3.7	19.0 ± 7.7	36.2 ± 2.4	38.9 ± 8.9	37.2 ± 7.6
	change (%)	+13.2 ^{n.s.}	-2.4* ^{n.s.}	change (%)	+2.2 ^{n.s.}	+0.53* ^{n.s.}	change (%)	+7.5 ^{n.s.}	-4.3* ^{n.s.}

Q_v : number of vessels per grid, L_v : length of vessels per tumor volume, V_v : volume of vessels per tumor volume, S_v : surface area of vessels per tumor volume, d: mean section diameter of vessels. Mean ± 1 S.D., Mann-Whitney U test where † $p < 0.05$, ‡ $p < 0.01$, and ‡ $p < 0.001$. *: Day 40, change from days 10, 18, and 20 respectively.

The proliferative index tended to be increased but not significantly in SU11657-treated tumors (Table 3).

Tumor cell differentiation. The tumors exhibited cells that stained positively for CgA and TH, confirming that the tumor cells were of neuroblastoma origin. TH immunoreactivity was most pronounced in the peripheral parts of the perivascular cuffs of viable tumor cells, and the number of TH-positive cells among SK-N-AS and SH-SY5Y cells increased significantly during therapy (Table 3). Expression of CgA protein was not quantified.

Plasma concentrations of VEGF-A, PDGF-BB, and SCF. The plasma concentrations of VEGF-A, PDGF-BB, and SCF were monitored during tumor progression. Animals that received SK-N-AS and SH-SY5Y grafts exhibited high concentrations of VEGF-A, whereas IMR-32-grafted animals had lower concentrations, similar to those in controls. By the end of the study (days 10–20), animals that were treated with SU11657 exhibited significantly lower plasma concentrations of VEGF-A compared with controls ($p < 0.001$; Fig. 2). The plasma concentrations were decreased even further after 40 d of treatment. The PDGF-BB and SCF concentrations first increased during therapy, but after treatment for 40 d, the plasma concentrations decreased. IMR-32 expressed the highest SCF concentrations. There were no significant differences between untreated control animals that did not have tumors and control animals that did not have tumors and received SU11657 treatment. The control animals without tumors showed significantly lower plasma concentrations of VEGF-A, PDGF-BB, and SCF compared with xenografted control animals. When the plasma concentrations were calculated per milliliter of tumor volume, an increase in VEGF-A, PDGF-BB, and SCF in the plasma at days 10, 18, and 20 of SU11657 therapy was seen. At day 40, the plasma concentrations of VEGF-A, PDGF-BB, and SCF per tumor volume were lower than those at days 10, 18, and 20 with all three cell lines.

DISCUSSION

Neuroblastoma is one of the most common malignant neoplasms in children. Tumor stage and patient age at diagnosis

correlate strongly with survival, in that age >1 y and high stages indicate a poor outcome. Biologic variables such as histopathology, ploidy, and amplification of MYCN oncogene (18,19) have also been shown to have a prognostic impact. There is also a correlation between high angiogenesis and a poor outcome in human neuroblastomas (20).

Changes in the net balance of angiogenesis inhibitors and growth factors directly affect vascularity, tumor growth, and metastasis (21). VEGF-A is considered the most important angiogenic factor (5). The biologic actions of VEGF are mediated through interactions with two high-affinity RTKs, namely VEGFR-1 and -2. VEGFRs, however, are not expressed exclusively by endothelial cells but also by, for example, hematopoietic stem cells and tumor cells (22). Inhibition of the kinase activity of VEGFRs suppresses tumor growth by inhibiting angiogenesis. For example SU5416, a small molecule designed to inhibit VEGFR-2 tyrosine kinase activity, has been shown to inhibit growth in a variety of experimental tumors (16,23). Another growth factor involved in angiogenesis is PDGF. This binds to three tyrosine-kinase receptors—PDGFR- α , PDGFR- β , and PDGFR- $\alpha\beta$ —which are expressed on endothelial cells under both normal (24,25) and pathologic conditions (26). C-KIT expression has been documented in a variety of human malignancies, and the kinase activity of c-KIT has been implicated in the pathophysiology of these tumors, including neuroblastoma (11). In view of the important roles of VEGF, PDGF, SCF, and FL and their receptors in tumor angiogenesis, it seems reasonable to expect that simultaneous inhibition of their signaling pathways may be more effective than inhibition of one pathway alone.

SU11657 is a selective multitargeted tyrosine kinase inhibitor with antitumor and antiangiogenic activity that is exerted by targeting class III/V RTKs, including 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 d, the tumors continued to grow but slowly. At day 40, the tumor

Table 3. Quantification of tumor dynamics and differentiation in the controls (vehicle only) and SU11657-treated tumors

Cell line	SK-N-AS			SH-SY5Y			IMR-32		
	Control (n = 11/11)	SU11657 (10 days) (n = 10/10)	SU11657 (40 days) (n = 9/9)	Control (n = 11/11)	SU11657 (18 days) (n = 10/10)	SU11657 (40 days) (n = 9/9)	Control (n = 11/11)	SU11657 (20 days) (n = 10/10)	SU11657 (40 days) (n = 9/9)
Tumor Volume (ml)	4.9 \pm 0.9	0.49 \pm 0.12	0.61 \pm 0.18	3.9 \pm 0.2	0.43 \pm 0.12	0.45 \pm 0.14	3.9 \pm 0.3	0.24 \pm 0.16	0.44 \pm 0.12
	change (%)	-90 \ddagger	+24 $^{*n.s.}$	change (%)	-89 \ddagger	+4.6 $^{*n.s.}$	change (%)	-93 \ddagger	+83 *†
Ki-67 (%)	24.5 \pm 14.8	30.0 \pm 15.3	27.6 \pm 8.4	47.0 \pm 10.7	48.2 \pm 5.7	52.2 \pm 11.9	46.1 \pm 9.3	49.3 \pm 11.1	52.1 \pm 12.0
	change (%)	+22 $^{n.s.}$	-8.0 $^{*n.s.}$	change (%)	+2.6 $^{n.s.}$	+8.3 $^{*n.s.}$	change (%)	+6.9 $^{n.s.}$	+5.7 $^{*n.s.}$
TH (%)	6.0 \pm 1.3	17.6 \pm 3.2	15.2 \pm 0.8	6.4 \pm 0.4	17.5 \pm 2.6	16.7 \pm 4.2	5.1 \pm 1.3	5.5 \pm 2.6	5.5 \pm 2.1
	change (%)	+193 \ddagger	-13.6 $^{*n.s.}$	change (%)	+173 \ddagger	-4.6 $^{*n.s.}$	change (%)	+7.8 $^{n.s.}$	-0.0 $^{*n.s.}$
TUNEL (%)	22.8 \pm 5.7	41.6 \pm 8.0	45.1 \pm 9.6	29.2 \pm 11.3	41.0 \pm 10.4	52.6 \pm 7.9	39.8 \pm 9.0	49.9 \pm 3.8	67.6 \pm 13.1
	change (%)	+83 \ddagger	+8.4 $^{*n.s.}$	change (%)	+40 \parallel	+28 $^{*n.s.}$	change (%)	+25 \parallel	+35 *†
VEGF- A (%)	17.6 \pm 3.2	24.2 \pm 7.2	24.5 \pm 4.9	20.3 \pm 6.1	29.8 \pm 4.0	29.1 \pm 3.7	12.4 \pm 0.9	15.7 \pm 2.3	13.9 \pm 2.9
	change (%)	+37.5 \ddagger	+1.2 $^{*n.s.}$	change (%)	+46.8 \parallel	-2.3 $^{*n.s.}$	change (%)	+26.6 $^{n.s.}$	-11.5 $^{*n.s.}$

Mean \pm 1 S.D. $\dagger p < 0.05$, $\parallel p < 0.01$, $\ddagger p < 0.001$, n.s.; not significant, * Day 40, change from day 10, 18, and 20 respectively.

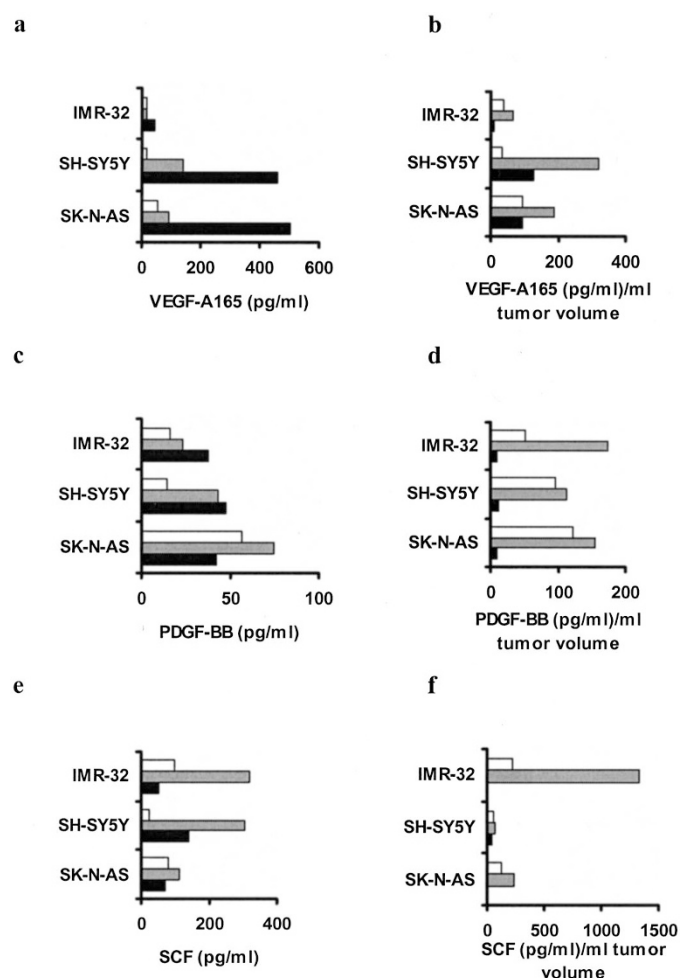


Figure 2. Plasma levels of VEGF-A (A), VEGF-A/mL tumor volume (B), PDGF-BB (C), PDGF-BB/mL tumor volume (D), SCF (E), and SCF/mL tumor volume (F) in the neuroblastoma cell lines SK-N-AS, SH-SY5Y, and IMR-32. ■, controls; ▒, SU11657 treatment for 10, 18, and 20 d, respectively; □, SU11657 treatment for 40 d.

volume had increased by 24% (SK-N-AS), 4.6% (SH-SY5Y), and 83% (IMR-32) compared with days 10, 18, and 20. The treated animals did not show any signs of toxicity. The fraction of apoptotic cells was significantly increased by therapy at days 10, 18, and 20. Apoptosis was increased to an even greater extent at day 40, whereas proliferation was unaffected. This increase in apoptosis can explain why the net growth of the tumor was reduced even though the proliferation was constant. SU11657 exhibited potent antiangiogenic activity in all experimental neuroblastomas. After 40 d of therapy, the tumor angiogenesis was reduced further, but this change was not significant. The reduction of angiogenesis induced by SU11657 is likely to be caused by interference with endothelial RTK signaling. However, the observed increase in tumor cell apoptosis may be either an indirect effect of angiogenesis inhibition or a direct effect on tumor cell signaling, promoting cell survival. Alternatively, potent RTK inhibition may perturb the suggested cross-talk between the tumor cell and endothelial cell compartments, blocking bilateral growth factors and survival factors (27,28). These three suggested mechanisms of action can be addressed further by *in vitro* assays with

SU11657. It is concluded that SU11657 is a potent inhibitor of experimental neuroblastoma growth and angiogenesis.

Plasma concentrations of human VEGF-A, PDGF-BB, and SCF were assayed. Several other neuroblastoma cell lines have been reported to release VEGF-A to the culture medium (29). In our study, we confirm that VEGF-A is produced by the neuroblastoma cells both *in vitro* and *in vivo*. Treatment with SU11657 reduced the plasma concentrations of VEGF-A in all groups. In contrast, there was an increase in the PDGF-BB and SCF concentrations during therapy. It is interesting that the animals that were grafted with MYCN-amplified IMR-32 exhibited low circulating concentrations of VEGF-A but significantly higher concentrations of SCF compared with controls. Also, IMR-32 was the cell line that exhibited the smallest increase in apoptosis during therapy. This might be explained by the observation that SCF is produced by neuroblastoma cells and protects them from apoptosis *via* an autocrine loop (12). However, looking at the plasma concentrations per milliliter of tumor volume, we saw an up-regulation of VEGF-A, PDGF-BB, and SCF in plasma at days 10, 18, and 20 of SU11657 therapy. At day 40, the plasma concentration per milliliter of tumor volume had decreased compared with days 10, 18, and 20 with all three cell lines. This phenomenon could be due to release of growth factors from apoptotic and necrotic cells, *i.e.* dying tumor cells. During long-term treatment, *e.g.* 40 d, the plasma levels decreased. This could indicate that the tumors elaborate other pathways to attract new vessels, that the growth factors that were liberated to the circulation as a result of necrosis have been cleared from the circulation, or that the functional tumor burden has 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 d of treatment. This could be due to a decreased number of viable tumor cells after long-term therapy.

We demonstrated for the first time, by immunohistochemistry, that the three studied neuroblastoma cell lines 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 areas of apoptosis and necrosis, except for SH-SY5Y. C-KIT expression was less frequent in SH-SY5Y, where only 40% of the control tumors expressed the receptor. The expression of the receptors decreased during therapy, and after 40 d 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. PDGF receptor expression in the stroma has been reported in a broad range of solid tumors (30). IMR-32 tumors are stroma-rich, but there was no receptor immunoreactivity after 40 d of therapy. This may explain why inhibition of PDGF receptors was effective in IMR-32 tumors. Thus, our experimental data warrant an examination of the expression of class III/V RTKs in human neuroblastomas with different clinical courses.

In conclusion, by using well-documented experimental models for human neuroblastoma, we show here that SU11657 reduces the tumor growth rate and angiogenesis, including

MYCN-amplified cell lines, at a well-tolerated dose. Combining these results, we suggest that SU11657 may be a candidate for neuroblastoma therapy in children.

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