

Potent antitumor effects of ZD6474 on neuroblastoma via dual targeting of tumor cells and tumor endothelium

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Abstract

Among children with relapsed or refractory neuroblastoma, the prognosis is poor and novel therapeutic strategies are needed to improve long-term survival. As with other solid tumors, high vascular density within neuroblastoma is associated with advanced disease, and therapeutic regimens directed against the tumor vasculature may provide clinical benefit. The receptor tyrosine kinase RET is widely expressed in neuroblastoma and is known to activate key signal transduction pathways involved in tumor cell survival and progression including Ras/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt. We investigated the effect of dual targeting of tumor cells and tumor endothelium with ZD6474, a small-molecule tyrosine kinase inhibitor of vascular endothelial growth factor (VEGF) receptor 2, epidermal growth factor receptor, and RET. ZD6474 inhibited the phosphorylation of RET in neuroblastoma cells and had a direct effect on tumor cell viability in seven neuroblastoma cell lines. In a human neuroblastoma xenograft model, ZD6474 inhibited tumor growth by 85% compared with treatment with vehicle alone. In contrast, no significant inhibition of tumor growth was observed after treatment with bevacizumab,

an antihuman VEGF monoclonal antibody, or the epidermal growth factor receptor inhibitor erlotinib, either alone or in combination. Immunohistochemical analysis showed that ZD6474 treatment led to an increase in endothelial cell apoptosis along with inhibition of VEGF receptor-2 activation on tumor endothelium. In conclusion, dual targeting of tumor cells, potentially through RET inhibition, and tumor vasculature with ZD6474 leads to potent antitumor effects. This approach merits further investigation for patients with neuroblastoma. [Mol Cancer Ther 2008;7(2):418–24]

Introduction

Neuroblastoma is the most common cancer in the first year of life and the most common solid tumor of childhood. There are limited effective treatment options for children with advanced disease, and mortality remains high for these patients. Standard therapy for advanced disease includes chemotherapy, surgery, and radiation. Patients who do not respond to these modalities may undergo high-dose chemoradiation followed by bone marrow transplant (1, 2), which seems to provide some control of disease progression but is complicated by significant morbidity and mortality. Neuroblastoma has been described as a highly angiogenic tumor with poor prognosis linked to vascular index (3), suggesting that this tumor type may respond to antiangiogenic treatment. Targeting the vascular network of neuroblastoma tumors with antiangiogenic agents has only recently been investigated, and a variety of approaches are being used (4).

Antiangiogenic tumor treatments can target various aspects of endothelial cell physiology. Vascular endothelial growth factor (VEGF) has been characterized as a critical mitogen regulating the growth, proliferation, and migration of endothelial cells and is a key regulator of angiogenesis (5). Further, its expression has been linked to prognosis for a number of human tumors (6). VEGF acts primarily by binding to one of its cognate receptors (VEGFR1, VEGFR2, and VEGFR3) on endothelial cells, leading to autophosphorylation of tyrosine residues and subsequent activation of intracellular signaling, such as the mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways (7–9). The targeting of the VEGF signaling pathway in tumor endothelium has emerged as a promising approach to suppression of neuroblastoma tumor growth (10–17). In addition to VEGF, other growth factor signaling pathways are implicated in tumor progression. Expression of epidermal growth factor (EGF) or its receptors has been shown to correlate with angiogenesis and expansion of several human tumors (7–9, 18–22). Similar to VEGFR2, epidermal growth factor receptor (EGFR) signaling is also mediated by the mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt

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pathways and has recently been shown to play a role in tumor angiogenesis (19).

The RET proto-oncogene encodes a receptor tyrosine kinase known to be expressed on neuronal and neural crest-derived cells (23, 24). Activation of RET by its soluble ligand, glial-derived neurotrophic factor (GDNF), stimulates signal transduction pathways, including phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase, and modulates cell proliferation, differentiation, migration, and survival (25). Several studies have implicated RET as an important molecule in the development and progression of neuroblastoma. RET is highly expressed on neuroblastoma cells (23), and transgenic mice overexpressing RET develop neuroblastomas (26). Furthermore, expression of constitutively activated RET in neuroblastoma cells results in enhanced tumor metastasis (27). However, whether RET tyrosine kinase inhibitors have activity against neuroblastoma has not been established. Because it is likely that drugs with dual activity against tumor cells and tumor-associated endothelial cells will have greater efficacy than drugs targeting either single component, we sought to determine the antitumor activity of ZD6474, a potent, low molecular weight inhibitor of VEGFR2, EGFR, and RET on neuroblastoma (28).

In the present report, we evaluated neuroblastoma cell lines and found that RET is expressed on neuroblastoma cells, and activation of RET is inhibited by ZD6474. We show that ZD6474 has direct antitumor activity on neuroblastoma cells *in vitro*. Moreover, the growth of neuroblastoma xenografts is significantly inhibited by ZD6474 whereas bevacizumab and erlotinib, inhibitors of the VEGFR and EGFR pathways, respectively, did not hinder tumor growth. These results show that therapies directed against neuroblastoma cells, potentially through RET inhibition, and the tumor vasculature may be a successful strategy for the treatment of this disease.

Materials and Methods

Cells

SK-N-SH cells expressing luciferase were kindly provided by Dr. William Weiss (University of California San Francisco, San Francisco, CA) and SK-N-MC cells were obtained from American Type Culture Collection. SK-N-SH and SK-N-MC cells were maintained in DMEM with 10% fetal bovine serum (Hyclone), glutamine, penicillin, and streptomycin (BioWhittaker). SK-NAS, NGP, and CHP-134 were kindly provided by Dr. John Maris (The Children's Hospital of Philadelphia, Philadelphia, PA), and LA1-55-N, SH-SY5Y, and SH-EP were kindly provided by Dr. Susan Cohn (Children's Memorial Hospital, Chicago, IL). Cell lines were maintained in RPMI with 20% fetal bovine serum, glutamine, penicillin, and streptomycin.

Western Blot Analysis

To evaluate RET expression, neuroblastoma cells were seeded onto individual wells of six-well plates at 70% confluency, incubated overnight, and lysed in lysis buffer

[20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L sodium chloride, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1% aprotinin, 20 μ mol/L leupeptin, and 0.15 units/mL aprotinin]. Protein (80 μ g) was separated by SDS-PAGE and blotted onto nitrocellulose. Membranes were incubated overnight at 4°C with a 1:200 dilution of rabbit anti-human RET antibodies (Santa Cruz Biotechnology) in blocking solution. To evaluate expression of EGFR and VEGFR2 on neuroblastoma cells, equal numbers of SK-N-SH or SK-N-MC cells were incubated overnight in OptiMem with 1% fetal bovine serum and then lysed in SDS sample buffer. Lysates were loaded onto 6% Tris-glycine gels, separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with a 1:1,000 dilution of rabbit anti-human EGFR antibodies (Cell Signaling) or a 1:700 dilution of rabbit anti-human VEGFR2 antibodies (Cell Signaling). To evaluate RET expression, SK-NAS cells were plated at 70% confluency and incubated overnight. Cells were treated with or without 10 μ mol/L ZD6474 (AstraZeneca) for 15 h and stimulated with media containing GDNF (50 ng/mL; PeproTech, Inc.) alone or in the presence of 10 μ mol/L ZD6474 for 10 and 30 min. Protein (50 μ g) was separated by SDS-PAGE and blotted onto nitrocellulose. Membranes were incubated overnight at 4°C with a 1:500 dilution of rabbit anti-human phospho-RET antibodies (Cell Signaling).

Cell Viability Assay

Neuroblastoma cells were plated onto 96-well plates at a density of 2,000 per well and incubated overnight. Medium was removed and 100 μ L of control media or media containing ZD6474 (0.1, 1, and 10 μ mol/L; AstraZeneca), bevacizumab (1 and 10 μ g/mL; Genentech, Inc.), or erlotinib (1 and 10 μ mol/L; OSI Pharmaceuticals, Inc.) were added. After 5 days, 50 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol-tetrazolium bromide (MTT; Sigma-Aldrich) were added to each well. Following a 2-h incubation, medium was removed, 100- μ L DMSO was added to each well, and plates were read using a μ Quant plate reader (Bio-Tek, Inc.) set at 570 nm.

Mouse Tumor Models

All mouse protocols were approved by the Children's Hospital Institutional Animal Care and Use Committee. Eight-week-old male athymic (*nu/nu*) mice were obtained from Charles River Laboratories. Mice were anesthetized with isoflurane (Baxter), and 15×10^6 SK-N-SH cells were injected into the s.c. flank region. After 4 weeks, mice bearing tumors between 5 and 200 mm³ were selected and randomized into treatment groups averaging 100 mm³. Treatment was started on the day of randomization. Drugs were administered at doses and schedules previously shown to be highly efficacious in tumor models and were at, or below, the respective maximum well tolerated dose in nude mice. The dosing schedules were ZD6474, 30 mg/kg/d p.o.; bevacizumab, 3 mg/kg i.p. twice weekly; and erlotinib, 75 mg/kg/d p.o. Animals were treated for 4 weeks. Tumor length and width were determined twice weekly, and tumor volume (TV) was calculated using the following formula: TV = length \times width² \times 0.52.

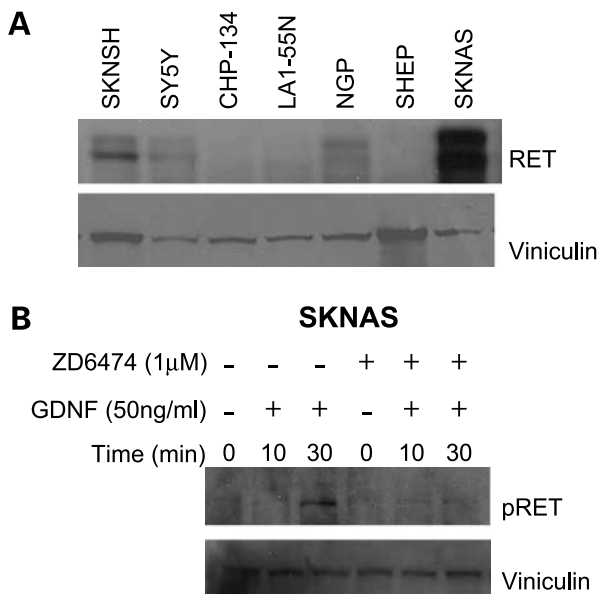


Figure 1. Expression of RET on neuroblastoma cell lines. **A**, neuroblastoma cell lines were evaluated for RET expression by Western blot. SK-N-SH, SY5Y, NGP, and SK-NAS cells produce detectable levels of RET. **B**, phosphorylation of RET is inhibited by ZD6474. SK-NAS cells were pretreated with 10 μ mol/L ZD6474 and then stimulated with GDNF (50 ng/mL) for 10 and 30 min alone or in the presence of ZD6474. Whole-cell lysates were evaluated by Western blot (pRET antibodies from cell signaling).

Immunohistochemistry

Mice were euthanized and then tumors were harvested and fixed in neutral buffered formalin for paraffin sections. Formalin-fixed, paraffin-embedded tumor sections were deparaffinized by successive incubations in xylene, 95% ethanol, 90% ethanol, 70% ethanol followed by PBS. Epitopes were unmasked with 20 μ g/mL proteinase K in PBS at room temperature for 30 min and rinsed twice in PBS + 0.3% Triton X-100. The sections were immunostained with rat anti-CD31 monoclonal antibodies (1:50; PharMingen) overnight at room temperature followed by incubation for 2 h with goat anti-rat Alexa 594-conjugated secondary antibodies (1:500; Molecular Probes) and costained with anti-VEGFR2 (1:50; Cell Signaling), anti-EGF (1:50; Cell Signaling), or anti-phospho-VEGFR (1:50; Cell Signaling) antibodies followed by goat anti-rabbit Alexa 488-conjugated secondary antibodies (1:500; Molecular Probes) for 2 h at room temperature. Nuclei were labeled by brief washes in Hoechst dye (1 μ g/mL; Sigma-Aldrich).

For terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, following anti-CD31 immunostaining, sections were TUNEL stained using the Dead-End labeling kit (Promega) according to the manufacturer's instructions. In brief, sections were incubated for 15 min in equilibration buffer followed by incubation with fluoresceinated rdNTPs and terminal deoxynucleotidyl transferase for 60 min at 37°C. Reactions were stopped by incubation in 2 \times SSC for 15 min followed

by serial washes in PBS + 0.3% Triton X-100. Cell nuclei were stained with Hoechst dye (1 μ g/mL; Sigma-Aldrich). Images were obtained on a Zeiss fluorescence microscope (Carl Zeiss, Inc.) and analyzed with AxioVision 4.0 software (Carl Zeiss Vision).

Results

Neuroblastoma Cells Express RET, and Activation of RET Is Inhibited by ZD6474

To evaluate RET expression on neuroblastoma cells, whole-cell lysates from seven neuroblastoma cell lines were evaluated by Western blot analysis. As shown in Fig. 1A, SK-N-SH, SH-SY5Y, NGP, and SK-NAS cells were positive for RET expression. To determine whether ZD6474 inhibits RET activation in neuroblastoma cells, SK-N-SH cells were stimulated with 50 ng/mL of the RET ligand GDNF, alone or in the presence of ZD6474. Whereas phosphorylated RET was detected in tumor cells treated with GDNF, phosphorylated RET was undetectable in cells treated with GDNF in the presence of ZD6474 (Fig. 1B).

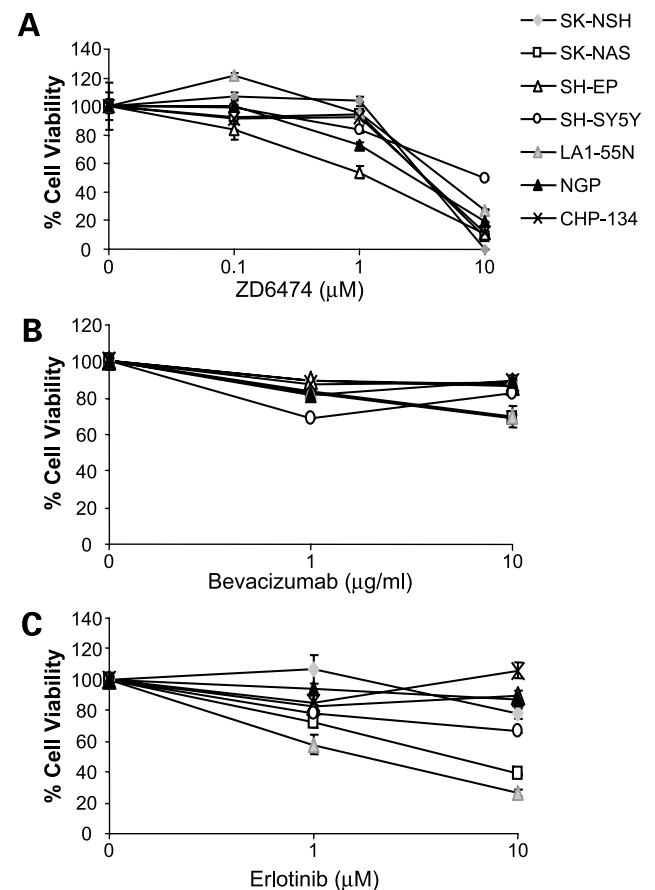


Figure 2. ZD6474 reduces cell viability of neuroblastoma cells. The effects of ZD6474 (**A**), bevacizumab (**B**), and erlotinib (**C**) on neuroblastoma tumor cell viability were evaluated by MTT assay. In five of seven cell lines, ZD6474 had $IC_{50} < 10$ μ mol/L. None of the cell lines were sensitive to bevacizumab, and erlotinib had $IC_{50} < 10$ μ mol/L in only two of seven cell lines.

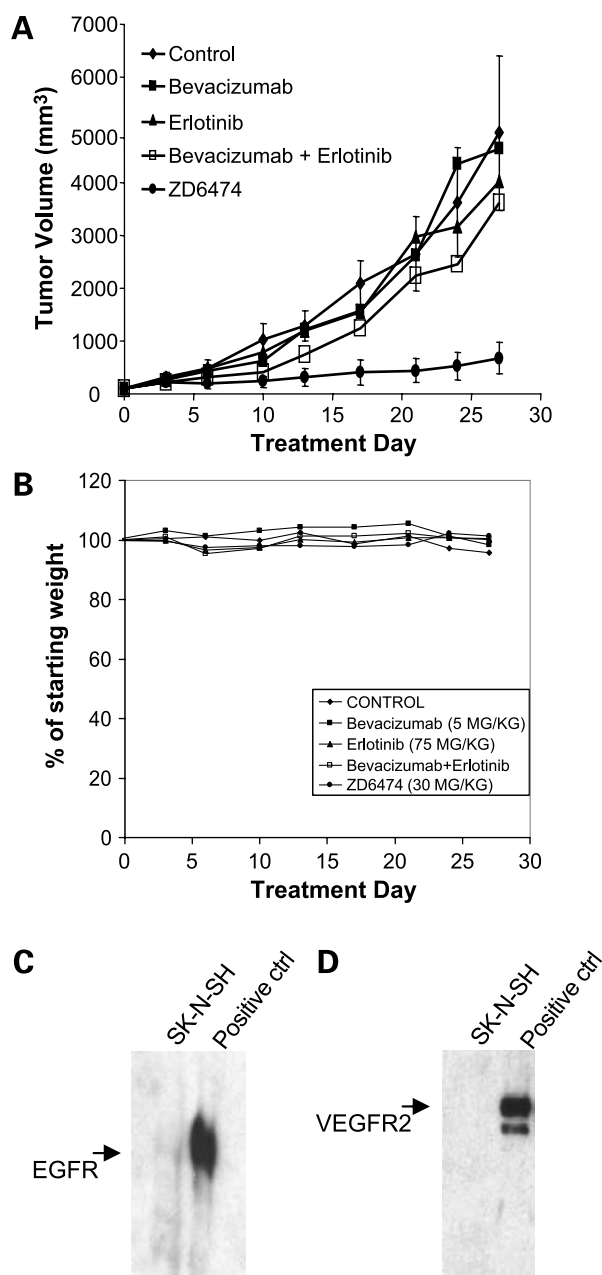


Figure 3. Neuroblastoma xenografts were inhibited by ZD6474 treatment. **A**, SK-N-SH neuroblastoma cells (15×10^6) were injected s.c. into the flanks of nude mice. After 4 wks, mice bearing tumors between 5 and 200 mm³ were selected and randomized into treatment groups averaging 100 mm³. Treatment was started on the day of randomization. ZD6474 and erlotinib were given by daily oral gavage as a suspension in 1% polysorbate. Bevacizumab (5 mg/kg) was given by s.c. injection biweekly. Tumor volumes and body weights (**B**) were obtained biweekly. Error bars for controls and ZD6474 treated mice are shown. **C** and **D**, SK-N-SH neuroblastoma cells lack expression of EGFR (**C**) and VEGFR2 (**D**) as determined by Western blot.

ZD6474 Has a Direct Effect on Neuroblastoma Cell Viability

To ascertain whether ZD6474 has a direct antitumor effect on neuroblastoma cells *in vitro*, we treated seven

neuroblastoma cell lines with control media or media containing physiologically relevant concentrations of ZD6474 (0.1, 1, or 10 $\mu\text{mol/L}$) for 5 days and evaluated cell viability by MTT assay. ZD6474 inhibited cell growth of SK-N-SH, SK-NAS, SH-EP, NGP, and CHP-134 cells with inhibitory concentration (IC_{50}) between 1 and 10 $\mu\text{mol/L}$ (Fig. 2A). Only two of seven cell lines, SH-SY5Y and LA1-55N, had an $\text{IC}_{50} \geq 10 \mu\text{mol/L}$. Next, we sought to determine the effect of EGFR and VEGFR2 pathway inhibitors on the viability of neuroblastoma cells. Neuroblastoma cell lines were incubated with control media or media containing bevacizumab (1 or 10 $\mu\text{g/mL}$) or erlotinib (1 or 10 $\mu\text{mol/L}$) for 5 days, and cell viability was evaluated by MTT assay. Bevacizumab did not have a significant effect on cell viability in any of the neuroblastoma cell lines (Fig. 2B) because an IC_{50} could not be achieved despite escalating dose. Among neuroblastoma cell lines treated with erlotinib, only LA1-55N and SK-NAS cells had an $\text{IC}_{50} \leq 10 \mu\text{mol/L}$ (Fig. 2C).

ZD6474 Inhibits Growth of Neuroblastoma Xenografts

Nude mice bearing SK-N-SH neuroblastoma xenografts were treated daily for 4 weeks with the small-molecule receptor tyrosine kinase inhibitor ZD6474 at 30 mg/kg. ZD6474 therapy caused 85% inhibition of tumor growth compared with control (vehicle alone) treated mice ($P < 0.05$; Fig. 3A). Tumor-bearing mice treated with bevacizumab, a monoclonal antibody targeting the proangiogenic protein VEGF, or treated with a small-molecule EGFR antagonist (erlotinib), either alone (0% and 14% inhibition, respectively; $P > 0.05$, not significant) or in combination (15% inhibition, $P > 0.05$, not significant), did not significantly inhibit tumor growth as compared with mice treated with vehicle alone. The inhibition of tumor growth was not attributable to systemic toxicity because no significant weight loss was seen in any of the treatment groups (Fig. 3B).

VEGFR2 and EGFR Expression on Neuroblastoma Cells

ZD6474 inhibits the activation of VEGFR2, EGFR, and RET. To explore whether the observed antitumor effect of ZD6474 was due to inhibition of VEGFR2 or EGFR activation on tumor cells, we evaluated the expression of these receptors on neuroblastoma cells. As determined by Western blot analysis, SK-N-SH cell lines lack EGFR and VEGFR2 (Fig. 3C and D). Human microvascular endothelial cells served as positive controls for EGFR and VEGFR2.

ZD6474 Inhibits VEGFR2 and EGFR Phosphorylation

Neuroblastoma tumors from mice treated with vehicle alone were harvested after 4 weeks of treatment, sectioned, and immunostained with antibodies against CD31, an endothelial specific marker, and with antibodies against VEGFR2 or EGFR. Expression of EGFR (Fig. 4A) and VEGFR2 (Fig. 4B) was detected almost exclusively on endothelial cells and not on the neuroblastoma tumor cells. Similar localization of EGFR and VEGFR2 was also found on the endothelium in tumors from mice treated with bevacizumab, erlotinib, or ZD6474 (data not shown).

Tumor sections from control mice and mice treated with ZD6474 or bevacizumab and erlotinib were further analyzed for VEGFR2 activation by immunohistochemistry with antibodies against CD31 and phospho-VEGFR2 (Fig. 4C). Tumors from bevacizumab/erlotinib-treated mice displayed persistent VEGFR2 phosphorylation on tumor endothelium. However, tumors harvested from mice treated with ZD6474 showed a significant reduction in CD31⁺ cells and a lack of phospho-VEGFR2 immunoreactivity.

ZD6474 Induced Tumor Endothelial Cell Apoptosis

Tumor apoptosis was determined by TUNEL reactivity and coimmunostaining with antibodies against CD31 (Fig. 5). Whereas control tumors displayed negligible apoptosis, tumors treated with ZD6474 revealed TUNEL-positive cells, which colocalized with CD31⁺ cells showing a vascular pattern of staining. In contrast, tumors harvested from mice treated with bevacizumab/erlotinib did not exhibit significant TUNEL-positive staining in tumor endothelium but did show TUNEL reactivity in some tumor cells (data not shown). Similarly, tumors from mice

treated with vehicle alone had no TUNEL-positive endothelial cells.

Discussion

In this study, we investigated the efficacy of dual targeting of neuroblastoma cells and tumor vasculature with ZD6474, an inhibitor of VEGFR, EGFR, and RET. Effective targeting of endothelial cells was observed both *in vitro* and *in vivo*. We observed that neuroblastoma cells express RET, and *in vitro* the majority of neuroblastoma cell lines including SK-N-SH are sensitive to ZD6474 at physiologically relevant concentrations. Further, we note that SK-N-SH cells do not express VEGFR2 or EGF, suggesting that the direct antitumor cell activity of ZD6474 observed *in vitro* may be due to RET inhibition. Our *in vivo* finding that ZD6474 significantly inhibits the growth of neuroblastoma xenografts implies that therapeutic regimens designed to target both tumor vasculature and tumor cells are an effective therapeutic strategy for this disease.

Expansion of tumor mass is critically dependent on tumor angiogenesis or the growth of new blood vessels into the

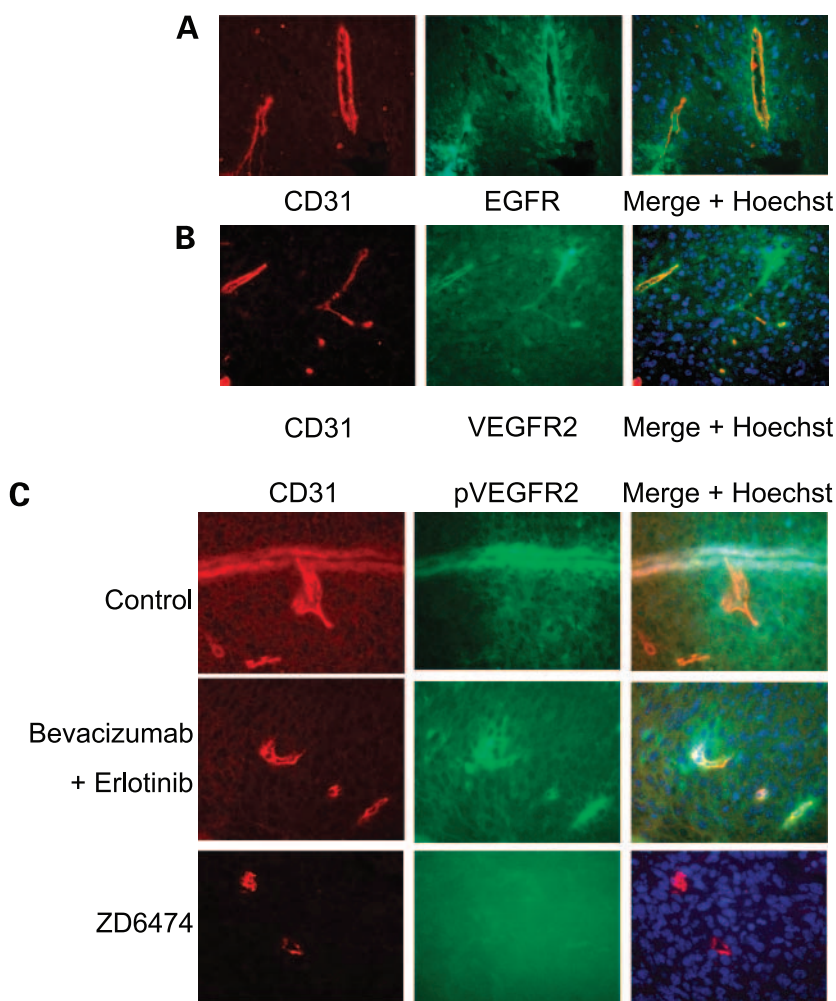
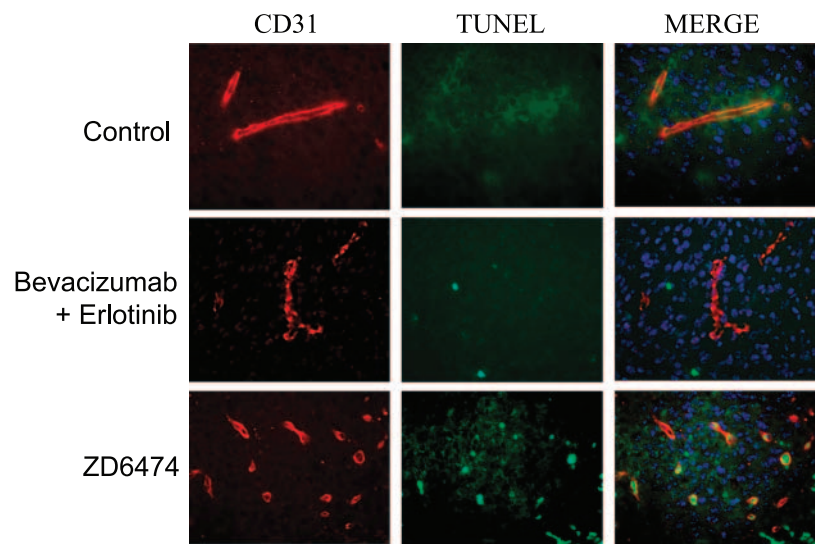


Figure 4. Expression of EGFR and VEGFR2 on neuroblastoma tumor endothelium. Neuroblastoma tumor sections were stained with anti-CD31 (red) and anti-EGFR (green; **A**) or anti-VEGFR2 (green; **B**). **C**, neuroblastoma sections from untreated, bevacizumab plus erlotinib-treated, or ZD6474-treated animals. Representative images show immunohistochemistry of CD31 (red) and phospho-VEGFR2 (green) and the colocalization (yellow) of phospho-VEGFR on endothelial cells.

Figure 5. Apoptosis of tumor endothelial cells in neuroblastoma xenografts. Tumor sections from untreated, bevacizumab plus erlotinib-treated, or ZD6474-treated animals were immunostained for CD31 (red) and TUNEL (green) to identify apoptotic endothelial cells (yellow).



tumor (29). Efforts to understand the mechanisms by which a tumor stimulates angiogenesis and maintains their blood supply have led to the development of antiangiogenic therapies. Targeting endothelial cell growth factor pathways has recently been validated as an effective addition to standard chemotherapeutic cancer treatments (30). ZD6474 has been shown to inhibit angiogenesis and reduce tumor growth in murine models of cancer. To determine the effect of ZD6474 on the vasculature within neuroblastomas, we evaluated tumors from mice treated with ZD6474 by immunohistochemistry. Our results indicate that ZD6474 effectively inhibited VEGFR phosphorylation on tumor endothelial cells, triggered endothelial cell apoptosis, and exerted an antiangiogenic effect on neuroblastoma xenografts. In contrast, bevacizumab failed to inhibit VEGFR phosphorylation and induce endothelial cell apoptosis. This difference may be due, at least in part, to the fact that bevacizumab inhibits VEGF signaling by binding human VEGF secreted by tumor cells and is unable to sequester host-derived murine VEGF produced by stromal cells within the tumor microenvironment. The ability of host-derived VEGF to sustain tumor angiogenesis has been proposed in previous studies (31). It is also feasible that ZD6474 altered the production of proangiogenic factors by tumor cells. In a recent report, activation of VEGFR1 in neuroblastoma cells was shown to result in stimulation of hypoxia-inducible factor 1 α and enhanced production of VEGF (32). Because our observations stem from work with a single neuroblastoma cell line (SK-N-SH) in a xenograft model, conclusions about the efficacy of bevacizumab against human neuroblastoma must be made with caution.

This study also examined the contribution of EGFR signaling to neuroblastoma tumor angiogenesis. Similar to VEGFR2, EGFR expression was localized primarily to tumor endothelial cells. However, in contrast with VEGFR2, EGFR blockade did not have any significant effect on tumor growth. These data imply that tumor endothelial EGFR signaling does not play a pivotal role in

neuroblastoma tumor growth in this model. These observations are supported by recent observations that another tyrosine kinase inhibitor, which more specifically blocks VEGFR1, VEGFR2, and VEGFR3, but not EGFR, potently inhibits neuroblastoma xenograft growth (33).

Prior studies have shown that neuroblastoma cells express RET and that constitutive activation of RET enhances *in vivo* growth and metastasis of neuroblastoma tumors (23, 27). Further, RET expression is commonly found in human neuroblastoma specimens (34). The role of RET inhibition in this neuroblastoma model is unclear. There was no clear correlation between expression of RET and inhibition of proliferation by ZD6474 *in vitro*, and we were unable to show *in vivo* tumor cell apoptosis. In addition, the possibility that some of the neuroblastoma cell lines tested may express VEGFR or EGFR and be susceptible to ZD6474 via these pathways cannot be excluded. In addition, inhibiting the RET pathway alone in SK-N-SH neuroblastoma cells *in vitro* with small interfering RNA does not seem to decrease proliferation (data not shown). It has been observed that the RET pathway is a key regulator of neuroblastoma differentiation *in vitro* (35). It is possible that inhibition of RET signaling in neuroblastoma cells by ZD6474 may more directly affect differentiation. Our data suggest, however, that the *in vitro* effects on neuroblastoma cells are not due to inhibition of EGFR or VEGFR, the other known targets for ZD6474. The lack of TUNEL-positive tumor cells after ZD6474 treatment implies that the direct effect of ZD6474 on neuroblastoma cells is primarily cytostatic.

In conclusion, we have shown that dual targeting of tumor vasculature and tumor cells with ZD6474 leads to potent antitumor effects, with tumor cell effects potentially mediated by RET inhibition. The identification of drugs with activity against tumor cells and tumor-associated endothelial cells may provide an important new therapeutic approach in the treatment of patients with neuroblastoma.

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