Inhibition of Multiple Vascular Endothelial Growth Factor Receptors (VEGFR) Blocks Lymph Node Metastases but Inhibition of VEGFR-2 Is Sufficient to Sensitize Tumor Cells to Platinum-Based Chemotherapeutics

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Abstract
Vascular endothelial growth factor receptors (VEGFR) have important roles in cancer, affecting blood and lymphatic vessel function as well as tumor cells themselves. We compared the efficacy of a VEGFR tyrosine kinase inhibitor, PTK787/ZK222584 (PTK/ZK), which targets the three VEGFRs, with blocking antibodies directed against VEGFR-2 (DC101) or VEGF-A (Pab85618) in a metastatic melanoma model. Although all inhibitors exerted comparable effects on primary tumor growth, only PTK/ZK significantly reduced lymph node metastasis formation. A comparable decrease in lymphatic vessel density following blockade of VEGFR-2 (DC101) or the three VEGFRs (PTK/ZK) was observed in the metastases. However, the functionality of lymphatics surrounding the primary tumor was more significantly disrupted by PTK/ZK, indicating the importance of multiple VEGFRs in the metastatic process. The antiangiostatic properties of PTK/ZK were confirmed in a breast carcinoma model. B16/B16 tumor cells express VEGF ligands and their receptors. Blockade of a VEGFR-1 autocrine loop with PTK/ZK inhibited tumor cell migration. Furthermore, the tumor cells also showed enhanced sensitivity to platinum-based chemotherapy in combination with PTK/ZK, indicating that autocrine VEGFRs are promoting tumor cell migration and survival. In summary, our results suggest that, in addition to blocking angiogenesis, combined inhibition of the three VEGFRs may more efficiently target other aspects of tumor pathophysiology, including lymphatic vessel functionality, tumor cell dissemination, survival pathways, and response to chemotherapeutic compounds. [Cancer Res 2008;68(5):1581–92]

Introduction
Metastatic spread of tumor cells is the underlying cause of most cancer-related deaths. Clinical and pathologic evidence shows that, for many human tumors, dissemination of cells via lymphatic vessels to local/regional lymph nodes is an early event in metastatic disease. Recently, there has been an increase in the number of mechanistic studies on tumor-associated lymphangiogenesis and lymphatic metastases (1, 2). Under physiologic conditions, lymphatic vessels in most adult tissues are quiescent. Results from various animal models suggest that lymphangiogenesis can be induced by solid tumors and may promote tumor spread (3). The most extensively studied signaling system promoting tumor lymphangiogenesis includes vascular endothelial growth factor (VEGF)-C and VEGF-D, and VEGF receptor-3 (VEGF-R3), their cognate receptor on lymphatic endothelium (4). An increasing number of clinicopathologic studies have shown a direct correlation between expression of VEGF-C or VEGF-D and metastatic tumor spread (5). These ligands, together with others implicated in tumor lymphangiogenesis, such as platelet-derived growth factors (6) and VEGF-A (7, 8), might enhance metastases by increasing the number of lymphatic vessels, thereby increasing contact between the invading cancer cells and the lymphatic endothelium. Moreover, VEGFR-2, the major blood vessel endothelial cell receptor for VEGF-A, has also been detected in collecting lymphatic vessels and capillaries undergoing active lymphangiogenesis (4). Because mature forms of VEGF-C, VEGF-D, and VEGF-A bind and activate VEGFR-2, and induce VEGFR-2/VEGFR-3 heterodimers (9, 10), VEGFR-2 might also influence lymphangiogenesis.

In addition to endothelial cell expression, VEGFRs are also expressed by tumor cells (11–14). Although the functions of VEGFR family members on tumor cells are not completely understood, the concomitant expression of VEGF and VEGFR suggests that these receptors might mediate biological effects in an autocrine fashion. Various approaches have been taken to interfere with the VEGF/VEGFR system, including antagonistic antibodies (15, 16), dominant-negative VEGFR mutants (17), recombinant soluble VEGFR proteins (18), and small-molecule tyrosine kinase inhibitors (TKI). Bevacizumab (Avastin, Genentech, Inc.), an anti–VEGF-A antagonist antibody, has been approved for use in combination with 5-fluorouracil (5-FU)-based chemotherapy for treatment of patients with metastatic colorectal cancer (19, 20). PTK787/ZK222584 (PTK/ZK; vatalanib, Novartis Pharma/Schering AG; ref. 21), a potent pan-VEGFR TKI, inhibits angiogenesis, tumor growth, and metastasis formation in experimental carcinoma models (22, 23). PTK/ZK has displayed activity in early clinical trials (24, 25).

Here, we have compared the effectiveness of PTK/ZK with that of an anti–VEGFR-2 blocking antibody (DC101, ImClone Systems;
ref. 16) and an anti–VEGF-A blocking antibody (Pab85618 or bevacizumab, anti-mouse and anti-human VEGF-A, respectively) in inhibiting tumor metastases. Our results show that blockade of VEGFR-2 signaling affects primary tumor growth but additional inhibition of VEGFR-1 and/or VEGFR-3 signaling is required for a significant reduction of lymphatic vessel functionality and tumor cell metastatic spread. Blockade of VEGFR-1 and VEGFR-2 on tumor cells inhibits migration and enhances sensitivity to cisplatin/oxaliplatin-induced apoptosis, respectively. These findings suggest that blockade of multiple VEGFRs directly affects the biological functions of both endothelial and tumor cells.

Materials and Methods

Cell culture. B16/B16 mouse melanoma cells (from Dr. J. Fidler, Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX) were cultured in MEM EBS (AMIMED), 5% FCS, 2% vitamin, 1% nonessential amino acids, and 1% sodium pyruvate; A549, A375, and MB-MDA435 (American Type Culture Collection) were cultured in RPMI 1640, 10% FCS, 1% l-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Lymphatic endothelial cells (LEC) were maintained on fibronectin-coated culture dishes (50 µg/mL) in microvascular endothelial cell growth medium (PromoCell, BioConcept AG).

Angiogenesis (chamber) assay. The assay was performed as described (23). Mice were treated with PTK/ZK (25, 50, and 100 mg/kg, p.o.), DC101 (5, 10, 21, and 42 mg/kg, i.p.), or Pab85618 (0.5, 1.5, and 5 mg/kg, i.p.) 4 to 6 h before chamber implantation. Dosing was once daily for 3 days for PTK/ZK, once on day 3 for DC101, and on day 2 for Pab85618. Four days after implantation, animals were sacrificed and the chamber was removed and weighed as described (23, 26).

Tumor models. The B16/B16 murine melanoma model was used (23). Mice were treated with either vehicle PEG300, PBS (5 mL/kg), PTK/ZK (p.o., once daily at 25, 50, and 100 mg/kg), DC101 (i.p., every 3 days at 10, 21, and 42 mg/kg), Pab85618 (i.p., every 2 days at 1.5 and 5 mg/kg), or an IgG isotype control. No significant loss of body weight occurred in treated animals. On day 21, mice were perfused with 1% paraformaldehyde as described (26). Cervical lymph node metastases were weighed and frozen for histologic analysis.

Frozen BN472 tumors were washed in 0.9% NaCl with 100 µg/mL gentamicin and placed in a Petri dish with Ham’s F-12 medium (100 µg/mL gentamicin, 10% FCS), and fragments of ~25 mm³ were transplanted in mammary fat pads of Brown Norwegian rats (Harlan). Treatment with PTK/ZK, given as described above, was initiated 1 day after transplantation and continued for 4 weeks. Lung metastases were determined by counting foci on the surface of lungs fixed in Bouin’s solution. Excised axial lymph nodes were weighed. The A549 non-small lung cancer cells (1.5 × 10⁵) were injected s.c. into NMRI nu/nu mice. Treatment started with a palpable tumor size of 20 mm². PTK/ZK was given p.o. daily at 100 mg/kg, and cisplatin was given i.p. every 3 days at 2 mg/kg. After 41 days, animals were sacrificed and tumors were excised and weighed.

Lymphatic vessel density quantification. Tumor cryosections were stained for either LYVE-1 or VEGFR-3 as above. Pictures encompassing six representative regions were taken at ×10 magnification (Zeiss Axiosplan). Areas of the counted regions were measured using the Openlab 3.1.5 software (Improvision).

Lymphangiography. Mice were anesthetized (Forene, Abbott AG) and 3 to 5 µL of Evans blue (3.5% in saline) were injected in the tumor center. Images were acquired 5 min after injection. The lymphatic network length (µm) connected to the primary tumor was quantified using computer-assisted imaging software (KS-400 Imaging System, Zeiss; ref. 27).

Immunoblot analysis. Treatment of cultures with growth factor was performed after serum starvation and 2 h of incubation with the compound. Immunoprecipitations were performed as described previously (28), and immunocomplexes were resolved by SDS-PAGE and analyzed by immunoblotting. To determine the effect of combination drug treatments on extracellular signal-regulated kinase 1/2 (ERK1/2) and AKT activation, B16/B16 cells were made quiescent overnight in serum-free medium and pretreated with PTK/ZK (1,000 nmol/L) for 1 h followed by treatment with the indicated concentration of cisplatin (Platinol, Bristol-Myers Squibb GmbH) in growth medium for 6 h at 37°C. Cells were lysed and proteins were analyzed by immunoblotting.

Proliferation and apoptosis assays. Subconfluent LECs (5 × 10⁵ per well) were seeded into 96-well plates for 24 h. For proliferation assays, they were serum starved (basal medium, 1% FCS) and then VEGF-A (20 ng/mL) or VEGF-C (400 ng/mL) was added in the presence or absence of PTK/ZK or bevacizumab. The antiproliferative effect of PTK/ZK and bevacizumab on LECs was evaluated using a bromodeoxyuridine (BrdUrd) ELISA kit, as previously described (28). For apoptosis assays, the medium was replaced with basic medium containing 0.5% FCS and VEGF-A (20 ng/mL) or VEGF-C (400 ng/mL) was added in the presence or absence of PTK/ZK or bevacizumab for 48 h. The percentage of cell death was measured using the YO-PRO assay (29). For the combination studies, baths (B16/B16, A375, and MB-MDA435) were seeded into 96-well plates and then incubated in the presence or absence of PTK/ZK (1,000 nmol/L) or DC101 (1 µg/mL) combined with increasing concentrations of cisplatin (0–10 µg/mL), oxaliplatin (0–10 µg/mL), or 5-FU (0–10 µmol/L) for 24 h at 37°C. The percentage of cell death and cell number was measured using the YO-PRO assay. Growing A549 cells were incubated with PTK/ZK (1,000 nmol/L) for 24 h followed by PTK/ZK combined with cisplatin (0–25 µg/mL) for an additional 24 h at 37°C. Cells were stained with Annexin V (Vybrant Apoptosis Assay Kit 2, Molecular Probes) and analyzed with a FACScan (Becton Dickinson).

Migration assays. B16/B16 cells (1 × 10⁵) in MEM EBS plus 0.5% FCS with or without PTK/ZK (0–1,000 nmol/L), IgG (1.25 µg/mL), or DC101 (1.25 µg/mL) were placed on 8.0-µm pore size membrane inserted in 24-well plates (Boyden chambers, Becton Dickinson Labware). MEM EBS plus 0.5% fetal bovine serum (FBS) with or without PTK/ZK (0–1,000 nmol/L), IgG (1.25 µg/mL), or DC101 (1.25 µg/mL) were placed in the bottom wells. After 6 h, cells that did not migrate were removed; cells that had migrated were stained with Hoechst 33342. Membranes were removed and mounted on slides and pictures were taken at ×5 magnification. Migrated cells were counted using the computer-assisted imaging software Imaris (Bitplane AG).

Results

The in vivo antitumor and antimetastatic effect of PTK/ZK, DC101, and an anti–VEGF-A antibody. The effective doses and optimal regimens to block VEGF-mediated responses for the multi-VEGFR TKI PTK/ZK, the anti–VEGF-R2 (DC101) and anti–VEGF-A (Pab85618) antibodies were determined as described in Supplementary Fig. S1. A dose-response curve was obtained for primary tumor growth, for weight of cervical lymph nodes, and for a VEGF-driven angiogenesis chamber model (23, 26). The optimal concentration for each compound was chosen based on complete inhibition of angiogenesis in the chamber model and the maximum inhibitory effect on primary tumor growth. DC101, dosed at 42 mg/kg, was more efficient in tumor growth inhibition compared with the 21 mg/kg dose, but at the higher dose, no additional effect was observed on angiogenesis or lymph node metastases weight (Supplementary Fig. S1). The concentrations used in the following experiments were as follows: PTK/ZK, 100 mg/kg; DC101, 42 mg/kg; and Pab85618, 5 mg/kg.

PTK/ZK, DC101, and anti–VEGF-A (Pab85618) were evaluated as antitumor and antimetastatic agents in the syngeneic orthotopic B16/B16 melanoma model, an aggressive metastatic subline derived from B16 cells (30). When B16/B16 cells are implanted i.d. in the ears of C57BL6 mice, primary tumors form (Fig. 1A). Seven to 10 days after tumor cell implantation, cervical lymph node metastases develop (Fig. 1B), with new blood vessel formation becoming evident in the same time frame. The optimal time for antitumor therapy is between 7 days (when there are no significant differences in primary tumor sizes) and 21 days, as subsequently...
Figure 1. Effect of blockade of VEGFRs on primary tumor and lymph node metastases in the B16/BL6 orthotopic melanoma mouse model and the BN472 orthotopic breast carcinoma model. A, B16/BL6 primary tumor growth measured at days 7, 14, and 21 after tumor cell inoculation. Points, mean; bars, SE. B, average weight of cervical lymph node metastases measured on day 21 following treatment between day 7 and day 21. Columns, mean; bars, SE. *, P < 0.05, treatment groups versus control group (one-way ANOVA and post hoc Holm-Sidak test). C, immunofluorescent detection of DC101 and Pab85618 in B16/BL6 metastases. Tumor sections from lymph node metastases were stained for CD31 (green) and incubated with either anti-rat (DC101 treated; top) or anti-rabbit (Pab85618 treated; bottom) secondary antibodies (red). Images captured for endothelial cells (CD31; green) were overlapped with images captured for DC101 or Pab85618; colocalization of the two blocking antibodies and CD31 is shown in yellow (merge). D, left, BN472 primary tumor growth measured at days 7, 14, 21, and 28 after transplantation of tumor fragments from donor rats in the mammary fat pads of Brown Norwegian rats. Points, mean; bars, SE. Average weight of axial lymph node metastases (middle) and number of foci of lung metastases (right) measured on day 28 following treatment between day 1 and day 28. Columns, mean; bars, SE. *, P < 0.05, treatment groups versus control group (one-way ANOVA and post hoc Holm-Sidak test).
the metastases become necrotic. The agents were given as described in Materials and Methods, and on day 21, the animals were sacrificed and primary tumors and lymph node metastases were analyzed (Fig. 1A and B). The growth of the primary tumor and metastases in mice treated with either PEG (vehicle control for PTK/ZK) or PBS (vehicle control for DC101 and Pab85618) was not statistically different and the data were pooled in a control group. Similarly, no difference in the effect of IgG versus vehicle control was observed (data not shown). Although statistically significant inhibition of the primary tumor was observed in mice receiving either PTK/ZK (50–63%), DC101 (48%), or Pab85618 (55%; Fig. 1A), only PTK/ZK treatment resulted in a significant reduction of the metastases weight (Fig. 1B). Furthermore, differences in the frequency of lymph node metastases were also observed, although they did not reach statistical significance. Ninety-five percent (38 of 40) of control mice had metastases, whereas in the PTK/ZK-, DC101-, and Pab85618-treated mice 79% (19 of 24), 88% (21 of 24), and 89% (16 of 18), respectively, had metastases.

To confirm that the antibodies reached the metastatic site, cryosections of lymph node metastases were stained for CD31 and incubated with secondary antibodies against rat (DC101) or rabbit (Pab85618; Fig. 1C). Double staining revealed that both DC101 and Pab85618 had diffused into the metastases and were localized around the blood vessels.

The antitumor and antimetastatic properties of PTK/ZK were also assessed in the BN472 orthotopic rat mammary cancer model (Fig. 1D). Similar to the B16/BL6 melanoma data, PTK/ZK treatment resulted in a significant dose-dependent reduction in the size of the primary tumor (Fig. 1D, left) and in the axial lymph node and lung metastases (Fig. 1D, middle and right). It was not possible to test the efficacy of DC101 or Pab85618 because these antibodies do not cross-react with rat VEGF-A or VEGFRs.

Taken together, these results suggest that, in the B16/BL6 model, blockade of multiple VEGFRs is more effective in blocking metastasis compared with individual blockade of VEGFR-2 or VEGF-A. Thus, it seems that additional inactivation of VEGFR-1 and/or VEGFR-3 by PTK/ZK may suppress tumor cell dissemination in addition to inhibiting primary tumor cell growth and lymph node metastases. To address whether these observations reflect the blockade of angiogenesis and lymphangiogenesis, we examined the effect of PTK/ZK, DC101, and Pab85618 on blood and lymphatic vessel density in the subsequent experiments.

Blockade of intratumoral angiogenesis and lymphangiogenesis is not sufficient to reduce metastatic tumor cell spread. Tumor cells enter the lymphatic system by inducing intratumoral lymphangiogenesis or by disrupting preexisting lymphatics in the surrounding tissue (1, 31). We first examined if spread of B16/BL6 tumor cells is accompanied by lymphangiogenesis in the primary tumor and lymph nodes. Blood and lymphatic vessels were visualized 7 and 21 days after tumor cell implantation by immunostaining with antibodies for CD31 and LYVE-1, respectively. In the primary tumor, few lymphatics were observed throughout the entire time course. By day 21, peritumoral lymphatics and, in larger tumors, some intratumoral lymphatics were present (data not shown). In normal lymph nodes, preexisting peripheral lymphatics and blood vessels were observed (Fig. 2A) and tumor cell spread was accompanied by an increase in intratumoral lymphangiogenesis (Fig. 2A, day 7). Lymphatic vessels with lumen-containing tumor cells were also detected (Fig. 2A, bottom). At day 21, lymphatic vessels were present but compressed toward the edge of the tumor (Fig. 2A).

Next, we investigated whether the different effects of PTK/ZK, DC101, and Pab85618 on tumor metastases could be attributed to their differential abilities to block tumor angiogenesis and lymphangiogenesis. Cryosections of primary tumors and metastases from treated animals were stained and the blood and lymphatic vessel density was quantified (Fig. 2B). Due to the high number of CD31-positive vessels in the ear, we could not distinguish vessels in the skin from vessels in the primary tumor. All three agents reduced the blood vessel density in the metastases (Fig. 2B, top). Treatment with either PTK/ZK or DC101 significantly reduced lymphatic vessel density in the metastases (Fig. 2B), whereas no effect was observed in the primary tumor (data not shown). Because Pab85618 had no effect on lymphangiogenesis (Fig. 2B) or metastatic tumor growth (Fig. 1B), it was not further analyzed.

The results show that lymphangiogenesis in the metastases can be significantly reduced to essentially the same extent with DC101 and PTK/ZK treatment (Fig. 2B), reflecting that lymphatics formed in the lymph nodes during the metastatic process are equally affected by both. Furthermore, the stronger effect of PTK/ZK compared with DC101 on weight of the metastases is unlikely to be due to its inhibition of angiogenesis (i.e., of nutrient and oxygen supply) because DC101 had even a stronger effects on angiogenesis in the metastatic lymph nodes than did PTK/ZK (Fig. 2B). Thus, we were prompted to examine the lymphatics that allow spread from the primary tumor.

Efficient blockade of lymphatic vessel functionality reduces metastatic tumor cell spread. It is well documented that enlarged, hyperplastic lymphatics are present at the periphery of most tumors (32, 33). The increased size of these vessels and the high interstitial fluid pressure within the tumor may facilitate access of tumor cells into the peripheral lymphatics. Therefore, we next examined the effect of PTK/ZK and DC101 on functionality of the lymphatic vessels connected to the primary tumor by measuring their ability to uptake fluid. Fourteen days after tumor cell implantation, a time when the difference in primary tumor size between control and treated mice was not yet significant, Evans blue was injected in the tumor center and images were acquired. The functional lymphatic network connected to the primary tumors was assessed by visual inspection (Fig. 2C) and image analysis (Fig. 2D). PTK/ZK (100 mg/kg), compared with DC101 (21–42 mg/kg), blocked lymphatic vessel functionality more efficiently, as measured by Evans blue dye uptake (Fig. 2C and D) and by lymph node metastatic spread (Fig. 1B; Supplementary Fig. S1). At 25 mg/kg, PTK/ZK had no significant effect on primary tumor growth and metastases (Supplementary Fig. S1) or on lymphatic vessel functionality (Fig. 2D). These data provide evidence that inactivation of VEGFR-2 alone does not impair lymphatic endothelium functionality and metastatic spread of tumor cells as well as blockade of all the VEGFRs. Compared with DC101, PTK/ZK treatment resulted in a strong inhibition of lymphatic functionality, leading to impaired tumor cell spread and a more significant inhibition of metastatic tumor growth in the lymph nodes.

In vitro effect of PTK/ZK and bevacizumab on isolated LECs. To examine potential differences in the role of VEGF-R2 and VEGF-R3 in the lymphatic endothelium, we used human LECs as an in vitro model. VEGF-R2, VEGF-R3, and podoplanin, a mucin-like glycoprotein expressed in lymphatic vessels, were detected in the LECs (Supplementary Fig. S2A), attesting to the lymphatic nature of the cells. Addition of VEGF-C and, to a lesser extent, VEGF-A increased VEGF-R3 tyrosine phosphorylation (Supplementary Fig. S2A).
Fig. S2B) and AKT and ERK1/2 activation (Supplementary Fig. S2C). Pretreatment with 1,000 nmol/L PTK/ZK blocked VEGF-C–induced and VEGF-A–induced VEGFR-3 phosphorylation (Supplementary Fig. S2B), which was accompanied by a blockade of intracellular signaling (Supplementary Fig. S2C, top). Pretreatment with the anti–VEGF-A blocking antibody bevacizumab resulted in an attenuation of VEGF-A but not VEGF-C–induced AKT and ERK1/2 phosphorylation (Supplementary Fig. S2C, bottom). We next examined the consequences of VEGFR activity on LEC survival and proliferation. Treatment of LECs with either VEGF-A or VEGF-C led to an increase in BrdUrd incorporation (Supplementary Fig. S2E), and coaddition of PTK/ZK (10–1,000 nmol/L) blocked the ability of both VEGF isoforms to stimulate proliferation (Supplementary Fig. S2E, left). Treatment with bevacizumab (25–100 ng/mL) reduced VEGF-A–stimulated but not VEGF-C–stimulated proliferation (Supplementary Fig. S2E, right). Furthermore, treatment of serum-starved LECs with either VEGF-A or VEGF-C increased their viability (Supplementary Fig. S2D). VEGF-A–induced survival was blocked by both PTK/ZK and bevacizumab in a dose-dependent manner; PTK/ZK addition also resulted in a significant decrease in

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**Figure 2.** Time course of lymphatic vessel growth and effect of PTK/ZK or DC101 on lymphatic density and functionality in B16/BL6 metastases. A, pictures of lymph nodes before implantation and on days 7 and 21 after tumor cell inoculation. B, cryosections of lymph nodes stained for CD31 (brown) and the lymphatic vessel marker LYVE-1 (red). C, lymphangiogenic outgrowth and tumor cell invasion into lymph nodes. D, blood and lymphatic vessel density was determined by counting the number of CD31-positive or VEGFR-3–positive vessels per mm² in a midsection of a lymph node metastasis, respectively. Columns, mean; bars, SE. *, P < 0.05 versus control (ANOVA with post hoc Holm-Sidak test). C, 14 d after tumor cell implantation, the functional lymphatic networks connected to the primary tumors were quantified as described in Materials and Methods. D, the length of the functional lymphatic network (μm) and the weight of lymph node metastasis in mice treated with vehicle, PTK/ZK (25 and 100 mg/kg), or DC101 (21 and 42 mg/kg) were quantified as described in Materials and Methods. *, P < 0.001 versus control (ANOVA with post hoc Holm-Sidak test).
VEGF-C–induced cell viability (Supplementary Fig. S2D). Interestingly, treatment of the VEGF-C–treated cultures with 1,000 nmol/L PTK/ZK strongly induced apoptosis. Although we can only speculate, the results suggest that LEC cultures became strongly dependent on VEGF-C for their survival during 48 h of VEGF-C treatment. In summary, these results show that both VEGF-A and VEGF-C influence LEC biology via activation of VEGFR-2 and VEGFR-3. Considering that, in vivo, tumors are likely to be exposed to different VEGF ligands, blockade of VEGF-A–induced signaling alone might not be sufficient to inhibit activation of the tumor-associated lymphatic endothelium.

**Expression of VEGF ligands and receptors in B16/BL6 tumor cells and metastases.** VEGFR is also expressed on tumor cells, raising the hypothesis that it can influence tumor cell biology (34). To evaluate if B16/BL6 tumor cells might be targeted by anti-VEGF/VEGFR therapy, we first investigated VEGF ligands and ligands in cultured tumor cells. VEGFR-1 and VEGFR-2, but not VEGFR-3, were detected in B16/BL6 cells by immunocytochemistry (Fig. 3A). Flow cytometry confirmed the immunocytochemical analysis and showed that VEGFR-2, but not VEGFR-1, was only detectable after a permeabilization step, suggesting an intracellular localization (Fig. 3B). These results, together with other reports describing an intracellular redistribution of VEGFR-2 after activation (35), suggest the presence of a VEGF-VEGFR autocrine loop in B16/BL6 cells.

Expression of VEGF ligands was then assessed. The 58-kDa VEGF-C precursor and 29-kDa polypeptide were detected in B16/BL6 cell lysates (Fig. 3C); the 21-kDa polypeptide that stimulates both VEGFR-2 and VEGFR-3 was not detectable. However, because it results from processing that occurs after ligand secretion, it might be present. B16/BL6 cells also express VEGF-B and VEGF-D and secrete high levels of VEGF-A (Fig. 3C).

We next examined the in vivo expression of VEGFRs in sections of B16/BL6 lymph node metastases. CD31 or LYVE-1 staining (Fig. 3D, green) was used to highlight the blood or lymphatic vasculature, respectively. Immunofluorescence staining with specific VEGF antibodies (Fig. 3D, red) revealed that vessels (Fig. 3D, Merge, yellow) and tumor cells surrounding the vessels (Fig. 3D, Merge, red) express VEGFR-1 and VEGFR-2 (Fig. 3D, top and middle). Expression of VEGFR-3 was restricted to the lymphatic endothelium (Fig. 3D, bottom, Merge, yellow) and was not detectable on tumor cells. These data indicate that the B16/BL6 tumor cells also express VEGFR-1 and VEGFR-2 in vivo and could respond to autocrine-produced VEGF ligands.

**Effect of PTK/ZK and DC101 on B16/BL6 cell migration.** We next tested for the presence of an autocrine VEGF/VEGFR loop in B16/BL6 cells. Treatment of cells with PTK/ZK, but not with DC101, caused a decrease in constitutive ERK1/2 phosphorylation with a maximum of inhibition between 15 and 20 min; neither inhibitor had an effect on AKT activation (Fig. 4A). To look for a biological effect of VEGFR signaling, we examined proliferation and migration. Neither inhibitor had an effect on cell proliferation as determined by BrdUrd incorporation (data not shown; ref. 26). B16/BL6 cell migration was examined in a Boyden chamber–based assay. Treatment with PTK/ZK blocked basal migration in a dose-dependent manner (Fig. 4B), whereas DC101 displayed only a modest, nonsignificant effect (Fig. 4C). These results suggest a specific role for VEGFR-1 in ERK1/2 activation and migration of B16/BL6 cells.

**PTK/ZK enhances tumor cell response to platinum-based chemotherapy.** Based on the reports that VEGF-A protects endothelial cells from chemotherapeutic drug-induced apoptosis (36), we assessed whether the VEGF/VEGFR autocrine loop might also influence B16/BL6 tumor cell chemosensitivity. Treatment of cultures with cisplatin (10 μg/mL), oxaliplatin (10 μg/mL), or 5-FU (10 μmol/L) led to a 30% to 50% decrease in cell number (Fig. 5A and B; Supplementary Fig. S3A, right) and killed 2% to 8% of the tumor cells (Fig. 5A and B; Supplementary Fig. S3A, left). Addition of PTK/ZK to cisplatin (5–10 μg/mL) or oxaliplatin (10 μg/mL), but not to 5-FU (Fig. 5A and B; Supplementary Fig. S3A, left), significantly increased cell death in a dose-dependent manner to 20% to 50%. It should be noted that treatment with PTK/ZK or DC101 alone induced a low level of apoptosis that was variable and dependent on cell density (data not shown). Statistical analyses indicated highly significant interactions between PTK/ZK and cisplatin or oxaliplatin (P < 0.001), suggestive of strong combination effects between the TKI and platinum-based chemotherapeutic drugs.

To assess the effect of VEGFR-2 blockade alone in chemosensitization, a similar experiment was performed with DC101. A comparable decrease in B16/BL6 cell number was observed when either PTK/ZK or DC101 was combined with cytotoxic drugs (Fig. 5A and B). Treatment of B16/BL6 cells with DC101 (1 μg/mL) killed 10% of the cells and addition of oxaliplatin (10 μg/mL) increased cell death to ~30% (Fig. 5B, bottom). Statistical analyses indicated significant interactions between the two drugs (P = 0.045), suggestive of combination effects. Taken together, these results suggest that B16/BL6 cells are dependent on VEGFR-2 activity for survival and that this autocrine loop protects the tumor cells from platinum-based chemotherapeutics.

To search for the mechanism underlying the chemoprotective effect of VEGFR-2, we examined the combinatorial effect of PTK/ZK and cisplatin on AKT and ERK1/2 activity in B16/BL6 cells. Treatment of tumor cells with cisplatin had no effect on AKT activity and, at low concentrations (up to 6 μg/mL), had no effect on phosphorylated ERK1/2 (P-ERK1/2) level. However, cisplatin at 10 μg/mL lowered ERK1/2 activation (Fig. 5C), decreased cell number by 50% (Fig. 5A, right), and induced low levels of cell death (5%; Fig. 5A, left). Importantly, coadministration of PTK/ZK to cisplatin lowered the threshold for the cisplatin-mediated inhibitory effect on ERK1/2 activity from 10 μg/mL to 4 to 6 μg/mL (Fig. 5C), suggesting that ERK1/2 activity may underlie the chemoprotective effect of the autocrine VEGFR-2 loop.

**Figure 3.** Expression of VEGFRs and VEGF ligands in B16/BL6 melanoma cells. A, immunofluorescent staining of VEGFRs in B16/BL6 cells. The specificity of immunostaining was shown by the lack of signal in the absence of the primary antibodies (Control). Bar, 50 μm. B, B16/BL6 cells were stained with either an anti–VEGFR-1 antibody (red), a phycoerythrin-conjugated anti–VEGFR-2 antibody (red), or an isotype-specific control (black) before (left) and after (right) a permeabilization step as described in Materials and Methods of the Supplementary Data and analyzed by flow cytometry. C, total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti–VEGF-C, anti–VEGF-B, and anti–VEGF-D antisera. Conditioned medium was collected from B16/BL6 cells plated in either basal medium or growth medium, and VEGF-A secretion was measured using an ELISA kit as described in Materials and Methods. D, immunofluorescent detection of VEGFRs in B16/BL6 lymph node metastases. Tumor sections were double stained for VEGFR-1 (red, top) and CD31 (green, top); VEGFR-2 (red, middle) and CD31 (green, middle); or VEGFR-3 (red, bottom) and LYVE-1 (green, bottom). The images captured for endothelial cells (CD31 and LYVE-1; green) were overlapped with images captured for VEGFR-1, VEGFR-2, and VEGFR-3 (Merge, yellow).
To provide evidence supporting a role for VEGFR-2 in chemosensitization, we assessed the interaction of PTK/ZK with chemotherapeutics in additional human tumor cell lines: the VEGFR-2–positive and VEGFR-3–positive MB-MDA435 mammary carcinoma cells (Fig. 6 A) and A549 non–small lung carcinoma cells (Fig. 6 C) and the VEGFR-3–expressing A375 melanoma cells (Fig. 6 B). These tumor cell lines express multiple VEGF isoforms (Supplementary Fig. S4; ref. 37), making it likely that an autocrine VEGF/VEGFR loop is present in each. Treatment of MB-MDA435 and A375 cell lines with cisplatin (10 μg/mL; Fig. 6 A and B, right) or 5-FU (10 μmol/L; Supplementary Fig. S3 B and C, right) reduced cell number by 30% to 60%. Cisplatin killed 10% to 30% of the tumor cells [Fig. 6 A and B (middle)] and A549 cell death from 13% to 16% (Fig. 6 C, right). As observed for B16/BL6 cells, 5-FU had no effect on viability of MB-MDA435 and A375 tumor cell lines (Supplementary Fig. S3 B and C, left); its activity was not examined in the A549 cells. Addition of PTK/ZK to cisplatin (10 μg/mL) increased MB-MDA435 cell death from 30% to 45% (Fig. 6 A, middle) and A549 cell death from 13% to 16% (Fig. 6 B, right), whereas coaddition of PTK/ZK did not further increase the effect of cisplatin on viability of the VEGFR-2–negative A375 tumor cells (Fig. 6 B, middle). Statistical analyses indicated significant interactions between PTK/ZK and cisplatin (P = 0.005 and 0.013, respectively) in MB-MDA435 and A549 cells, suggestive of combination effects between the two drugs. In summary, addition of PTK/ZK to platinum-based drugs resulted in increased tumor cell death in the VEGFR-2–positive B16/BL6, MB-MDA435, and A549 cells, but not in the VEGFR-3–positive but VEGFR-2–negative A375 cells, suggesting that the autocrine activation of VEGFR-2, but not VEGFR-1 or VEGFR-3, has a role in tumor cell chemoresistance.

The in vivo effects of treatment with PTK/ZK and cisplatin were also examined in the A549 human lung cancer model. Importantly, there was a significant inhibition in tumor area and weight in animals treated with the combination of PTK/ZK and cisplatin compared with either drug alone (Fig. 6 D).

**Discussion**

VEGFRs have important roles in cancer, affecting blood and lymphatic vessel functionality as well as tumor cells themselves. The present study was undertaken to assess the effect of selective VEGFR-2 and VEGF-A inhibitors (DC101 and Pab85618) and PTK/ZK, a multiple VEGFR inhibitor, on the growth of primary tumor and lymph node metastases in the B16/BL6 melanoma model. We show that combined inhibition of multiple VEGFRs efficiently targets various aspects of tumor pathophysiology, including lymphatic vessel functionality and tumor cell dissemination. We also address the role of VEGFRs on tumor cells and provide evidence that autocrine activity of VEGFR-1 and VEGFR-2 has a role in tumor cell migration and chemoresistance, respectively.

Our results show that selective blockade of VEGFR-2 or VEGF-A is sufficient to abrogate primary tumor growth, but additional inhibition of VEGFR-1 and VEGFR-3 with PTK/ZK is required for a significant reduction in the metastatic spread of the melanoma cells. PTK/ZK was also very efficient in blocking metastases in the BN472 rat mammary carcinoma model (shown here) and in the RENCA renal cell carcinoma model (22). Moreover, it has previously been shown that, in the VEGF-C ectopically expressing MB-MDA435 breast tumor...
model, combination of anti–VEGFR-2 and anti–VEGFR-3 antibodies blocked metastases more efficiently than either antibody alone (38).

Lymphatic vessel endothelium expresses not only VEGFR-3 but also VEGFR-2 (39), raising the possibility that this receptor might also stimulate LEC proliferation and induce lymphangiogenesis. Indeed, VEGFR-2 activation seems to be sufficient to promote tumor lymphangiogenesis in some tumor models (2). Isolated LECs express both VEGFR-2 and VEGFR-3 and, in agreement with other studies (40), show a proliferative and survival response to VEGF-A and VEGF-C. Furthermore, the effects of VEGF-A on cultured LECs were inhibited by the VEGF-A blocking antibody. In vivo, however, this antibody was unable to block lymphangiogenesis or metastatic spread, suggesting that activation of the lymphatic VEGFRs occurs in response to VEGF-C and/or VEGF-D.

Importantly, our studies show that simultaneous inhibition of all VEGFRs impaired functionality of the lymphatic vessels connected to the primary tumor and this effect positively correlated with a decrease in lymph node metastases weight. VEGFR-2 blockade was able to lower lymphangiogenesis in the lymph nodes, but this was not sufficient to block metastasis, suggesting that preexisting

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**Figure 5.** PTK/ZK sensitizes B16/BL6 to platinum-based chemotherapy. A, growing B16/BL6 cells (5,000 per well) were incubated in the presence or absence of PTK/ZK (1,000 nmol/L) combined with increasing concentrations of cisplatin (0–10 μg/mL) for 24 h at 37°C. Cell death and cell number were measured using the YO-PRO assay as described in Materials and Methods. Columns, mean; bars, SE. *, P < 0.01 versus 0 μg/mL cisplatin (two-way ANOVA and Holm-Sidak test). B, growing B16/BL6 cells (10,000 per well) were incubated in the presence or absence of PTK/ZK (1,000 nmol/L) or DC101 (1 μg/mL) combined with oxaliplatin (10 μg/mL) for 24 h at 37°C. Cell death and cell number were measured using the YO-PRO assay. Columns, mean; bars, SE. *, P < 0.01 versus 0 μg/mL oxaliplatin (one-way/two-way ANOVA and Holm-Sidak test). C, B16/BL6 cells were pretreated with PTK/ZK for 1 h followed by 6-h treatment with the indicated concentrations of cisplatin in growth medium. Total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti–P-AKT, P-ERK, AKT, and ERK antibodies.
Figure 6. PTK/ZK sensitizes VEGFR-2–positive tumor cells to cisplatin treatment in vitro and in vivo. A and B, left, MB-MDA435 and A375 cells were stained with an anti–VEGFR-1 antibody (red), anti–VEGFR-2 antibody (green), anti–VEGFR-3 antibody (blue), or an isotype-specific control (black) as described in Materials and Methods of the Supplementary Data and analyzed by flow cytometry; right, growing MB-MDA435 or A375 cells were incubated in the presence or absence of PTK/ZK (1,000 nmol/L) combined with increasing concentrations of cisplatin (0–10 μg/mL) for 24 h at 37°C. Cell death and cell number were measured using the YO-PRO assay. Columns, mean; bars, SE. *, P < 0.001 versus 0 μg/mL cisplatin (one-way/two-way ANOVA and Holm-Sidak test). C, left, A549 cells were stained with an anti–VEGFR-1 antibody (red), anti–VEGFR-2 antibody (green), anti–VEGFR-3 antibody (blue), or an isotype-specific control (black) after a permeabilization step (0.3% Triton) as described in Materials and Methods of the Supplementary Data and analyzed by flow cytometry; right, growing A549 cells were incubated in the presence or absence of PTK/ZK (1,000 nmol/L) for 24 h followed by PTK/ZK combined with increasing concentrations of cisplatin (0–10 μg/mL) for an additional 24 h at 37°C. Cell death was measured using the Vybrant Apoptosis Assay Kit 2. Columns, mean; bars, SE. *, P < 0.001 versus 0 μg/mL cisplatin (one-way/two-way ANOVA and Holm-Sidak test). D, left, A549 primary tumor area measured every 3 d between day 7 and day 41 after tumor cell inoculation. Columns, mean; bars, SE. Right, average of tumor weight measured on day 41 following treatment between day 7 and day 41. Columns, mean; bars, SE. *, P < 0.05, treatment groups versus control group; #, P < 0.05, treatment PTK/ZK + cisplatin versus treatment with each agent alone (one-way ANOVA and post hoc Holm-Sidak test).
lymphatics surrounding the tumor are used by the B16/BL6 tumor cells to reach the lymph nodes. Furthermore, in contrast to results with PTK/ZK, blockade of VEGFR-2 alone had little effect on the functionality of lymphatic vessels connected to the primary tumor and this correlated with only a partial blockade of tumor cell spread to the lymph nodes. Taken together, our results suggest that inhibition of tumor angiogenesis, mainly via blockade of VEGFR-2, underlies the essentially equivalent in vivo efficacy of PTK/ZK, DC101, and PaB85618 on primary tumor growth. Importantly, blockade of the functionality of peripheral lymphatics surrounding the primary tumor, presumably via inhibition of the other VEGFRs, may underlie the efficient inhibition of metastatic spread observed with PTK/ZK treatment.

In addition to the important activity of VEGFs on endothelial cells, these growth factors might also stimulate the VEGFRs that have been detected on many tumor types (34). Indeed, in breast cancer, melanoma, and some leukemias, VEGF/VEGFR biological activities for autocrine signaling loops have been described (13, 41, 42). Furthermore, VEGFR-3 and VEGFR-1 have important roles in promoting lung adenocarcinoma cell invasion, and colorectal and pancreatic carcinoma cell migration, respectively (14, 43–45). Here, we show that B16/Bl6 cells express multiple VEGF ligands as well as VEGFR-1 and VEGFR-2. In vitro treatment with PTK/ZK, but not DC101, decreased ERK1/2 activity and strongly reduced B16/Bl6 cell migration. These data suggest that an autocrine VEGFR-1 activation loop promotes B16/Bl6 cell migration, agreeing with a recent report showing a specific role for VEGFR-1 in placenta growth factor (PIGF)-induced metastatic melanoma spread (46).

Finally, our work shows that autocrine VEGFR activity present in tumor cells mediates chemoprotection and survival. In vivo, combined treatment with PTK/ZK and cisplatin had a significant beneficial effect on tumor growth inhibition when compared with either agent alone. In vitro, combined treatment with platinum-based cytotoxic drugs and PTK/ZK was more effective in inducing death of the VEGFR-2–positive B16/Bl6, A549, and MB-MDA435 tumor cells than either therapy alone. Activation of both phosphatidylinositol 3-kinase and ERK1/2 can protect tumor cells from cisplatin-induced apoptosis (47, 48), and pharmacologic interruption of these survival pathways lowers the threshold for cisplatin-mediated lethality (49, 50). Our results show that treatment of B16/Bl6 tumor cells with PTK/ZK alone or in combination with cisplatin resulted in a decrease of ERK1/2 but not AKT activity, suggesting that the autocrine VEGFR activity–mediated chemoprotective effect occurs via activation of ERK1/2 signaling. DC101 also showed interactive effects with platinum-based chemotherapy in the B16/Bl6 melanoma cells. Interestingly, A375 melanoma cells, with low or undetectable levels of VEGFR-1 and VEGFR-2, were not sensitized by PTK/ZK to cisplatin-induced death, supporting the concept that VEGFR-2 has a specific role in the chemosensitization effect. Moreover, no increased cell death was observed when PTK/ZK was combined with 5-FU in either B16/Bl6 or MB-MDA435 tumor cell lines, suggesting that the sensitization to platinum-based chemotherapeutic drugs occurs via a specific mechanism.

In conclusion, an anti–VEGF-A-targeted antibody can block the VEGF-A contribution to angiogenic and lymphangiogenic signaling, and to tumor cell survival and spread, but is unable to restrict lymphatic spread promoted by PIGF and other members of the VEGF family. PTK/ZK, by blocking all three VEGFRs, inhibits angiogenesis, targets lymphatic vessel functionality and tumor cell dissemination, and, specifically via VEGFR-2 inhibition, enhances the tumor cell response to platinum-based chemotherapeutic drugs. Furthermore, combination of chemotherapeutic drugs and angiogenesis inhibitors has the potential to target both endothelial and tumor cells. Clinical trials addressing the ability of tumor VEGFR expression pattern to serve as a predictive factor for the outcome of combinatorial chemotherapeutics and VEGFR inhibitor therapy may be important in the future.

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