The Antitumor and Antiangiogenic Activity of Vascular Endothelial Growth Factor Receptor Inhibition Is Potentiated by ErbB1 Blockade

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Abstract

Purpose: Receptor tyrosine kinases of the ErbB family play important roles in the control of tumor growth. Vascular endothelial growth factor (VEGF) stimulates endothelial cell proliferation, enhances vascular permeability, and plays an important role in tumor vascularization. We evaluated the effects of selective VEGF receptor (VEGFR; PTK787/ZK222584) and ErbB (PKI166 and ZD1839) inhibitors on tumor growth and angiogenesis and asked whether additional therapeutic benefit was conferred by combination treatment.

Experimental Design: The antitumor activity of each inhibitor alone or in combination was assessed in human cancer models in immunocompromised mice. ErbB receptor expression and activation of downstream signaling pathway was evaluated in both tumor and endothelial cells.

Results: Both ErbB inhibitors significantly enhanced the antitumor activity of PTK787/ZK222584. In vitro, ErbB1 inhibition blocked VEGF release by tumor cells and proliferation of both tumor and endothelial cells. In an in vitro angiogenesis assay, epidermal growth factor (EGF) stimulated the release of VEGF by smooth muscle cells resulting in increased angiogenesis, a response blocked by administration of PTK787/ZK222584. Under basal condition, both ZD1839 and PTK787/ZK222584 blocked sprouting, likely via inhibition of an autocrine ErbB1 loop and VEGFR signaling, respectively, in endothelial cells. In conditions of limiting VEGF, EGF plays an important role in endothelial cell proliferation, survival, and sprouting.

Conclusion: We have shown that activation of ErbB1 triggers a plethora of effects, including direct effects on tumor and endothelial cells and indirect effects mediated via induction of VEGF release. Simultaneous blockade of ErbB1 and VEGFR pathways results in a cooperative antitumor effect, indicating that this combination may represent a valid therapeutic strategy.

The ErbB family of receptor tyrosine kinases has four members: ErbB1 (EGF receptor), ErbB2 (HER-2/neu), ErbB3 (HER3), and ErbB4 (HER4). These receptors play important roles in normal development and in cancer biology. In particular, ErbB1 and ErbB2 have been implicated in multiple types of human cancer, where they control proliferation, survival, and spread of metastatic cancer cells (1). Alterations in ErbB receptors have been correlated with more aggressive disease, lower rates of survival, and poor response to therapy (2). ErbB receptors are therefore considered valid targets for anticancer therapy. With respect to ErbB1, both antagonistic monoclonal antibodies and small-molecule tyrosine kinase inhibitors have shown clinical efficacy. Among these, ZD1839 (Iressa, Astra Zeneca Pharmaceuticals, Macclesfield, United Kingdom; ref. 3) and PKI166 (Novartis Pharma AG, Basel, Switzerland; ref. 4) are orally active ErbB1 and ErbB1/ErbB2 TK inhibitors, respectively. ZD1839 induced durable responses in patients with various tumor types (5). Both compounds, in addition to inhibiting tumor growth (6), have also been documented to have antiangiogenic effects (4, 7–9).

Angiogenesis, the formation of new vessels from an existing vascular network, is required to sustain tumor growth and invasion (10, 11). A link between angiogenesis and ErbB receptor signaling has been established. Dividing endothelial cells express functional ErbB1 (12, 13) and undergo apoptosis upon blockade of its activity (4). ErbB receptor inhibitors are effective in preventing the release of proangiogenic factors, such as vascular endothelial growth factor (VEGF), from tumor cells (14–16). VEGF exerts potent mitogenic and prosurvival effects on endothelial cells and has been shown to be a key factor in pathologic situations that involve neovascularization and changes in vascular permeability (17). The angiogenic signal is transmitted via cognate receptor tyrosine kinases (KDR and...
Ft-1), the VEGF receptors (VEGFR), located on the cell surface of the host vascular endothelium. Inhibition of VEGF signaling prevents tumor angiogenesis thereby starving tumors of essential nutrients and oxygen. Various approaches have been adopted to interfere with the VEGF/VEGFR system including antagonistic antibodies (18–20), dominant-negative VEGFR mutants (21), recombinant soluble VEGF proteins (22, 23), and small-molecule tyrosine kinase inhibitors. Among these, PTK787/ZK222584 (Vatalanib, Novartis Pharma/Schering AG, Berlin, Germany), a potent inhibitor of both VEGFRs, inhibited growth and angiogenesis in experimental carcinoma models (24, 25) and displayed activity in early clinical trials (26, 27). Although blockade of ErbB receptor signaling would be expected to inhibit both tumor growth and angiogenesis, we postulated that combination with a VEGF inhibitor may provide additional therapeutic benefit. This hypothesis is supported by in vivo preclinical data we have obtained using different agents to block ErbB and VEGFRs (patent no. WO02/41882; refs. 28, 29). In addition, prolonged inhibition of tumor growth (30) and vascularization (31, 32) was observed when agents targeting the VEGF axis were combined with antagonistic antibodies directed against ErbB1.

Here we have investigated the molecular mechanisms underlying the effect of combining selective ErbB and VEGF receptor inhibitors on the signaling pathways and biological functions of endothelial and tumor cells. We discuss the hypothesis that in the tumor microenvironment, blockade of ErbB1 signaling results in direct inhibition of tumor and endothelial cell proliferation and survival as well as indirect inhibition of VEGFR signaling via a decrease in VEGF release.

Materials and Methods

**Antibodies and reagents.** PTK787/ZK222584 was developed as a joint venture between Novartis Pharma and Schering. For in vitro assays, a 10 mmol/L stock solution of PTK787/ZK222584 and ZD1839 was prepared in DMSO and diluted in the optimal medium. For in vivo studies ZD1839, PKI166, and PTK787/ZK222584 were dissolved in 0.5% Tween, 5% DMSO in distilled water at the final concentration of 1 to 2 mg/mL.

The following antibodies were used: rabbit polyclonal anti-ErbB1 (1005) and anti-ErbB3 (C-17; Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERK1/2 and anti-phospho-ERK1/2 (Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti-ErbB2 antiseraum 21N (33), monoclonal anti-ErbB1 (120) and (101) (Santa Cruz Biotechnology), monoclonal anti-ErbB2 51 (34), monoclonal anti-ErbB3 (Ab-4; NeoMarkers, Fremont, CA), monoclonal anti-phosphotyrosine 4G10 (Novartis Pharma), monoclonal anti-CD31 (BD Bioscience, San Diego, CA), monoclonal anti-activated Caspase-3 (Oncogene Research Products, San Diego, CA), biotinylated anti-rabbit secondary antibody (Vector Labs, Peterborough, United Kingdom), and TRITC-conjugated anti-rabbit secondary antibody (The Jackson Laboratory, West Grove, PA). Growth factors used were: recombinant human epidermal growth factor (EGF) and heparin binding-EGF (Sigma, St. Louis, MO), recombinant human betacellulin (R&D System, Inc., Minneapolis, MN), recombinant human transforming growth factor-α (Upstate Biotechnology, Lake Placid, NJ), recombinant human heregulin (NeoMarkers), recombinant human basic fibroblast growth factor (bFGF, Life Technologies, Inc., Gaithersburg, MD), recombinant human platelet-derived growth factor (PDGF, Upstate), and recombinant dog VEGF125 (VEGF, Novartis Pharma).

Cell culture. The human umbilical vascular endothelial cells (HUVEC) and human pulmonary smooth muscle cell (HPASMC), purchased from Promo Cell (BioConcept AG, Allschwil, Switzerland), were cultured according to manufacturers’ instructions. The DU145 prostate cancer cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in MEM EBS (AMIMED, Allschwil, Switzerland) supplemented with 10% heat-inactivated FCS, 1% l-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin. HC11 mouse mammary epithelial cells were grown as described previously (35). NCI-H596 human adenosquamous lung cancer cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1% l-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin.

**Tumor models.** BALB/c nu/nu mice were obtained from Iffa Credo (L’Arbresle, France). Tumors were established by s.c. injection of DU145 or NCI-H596 cancer cells (minimum 1-2 × 10⁶ cells in 100 μL PBS). Tumors from donor mice were passaged s.c. as fragments three times before use. HC11 mouse mammary epithelial cells were transfected with oncogenic neuT and implanted as described previously (35). Three to 4 weeks after transplantation, mice showing mammary tumor growth of at least 10% during the previous 7 days were selected for efficacy studies. Animals were assigned to balanced groups, based on tumor size, so that means and SE were similar in each group (10-11 animals/group). Tumor growth and body weights were determined twice per week (35).

**Measurement of vascular endothelial growth factor concentration in plasma and tumor.** Repeated plasma samples were taken from several mice at different time points (days 0, 1, and 8) and collected in separation tubes (BD Microtainer PST LH, NJ), left at room temperature for 90 seconds and spun at 4°C for 7 minutes at 7,000 rpm. Tumors were homogenized (BIO 101, Fastprep) in 500 μL PBS + 0.1% bovine serum albumin and centrifuged. The concentration of human VEGF in the supernatants and plasma was measured using a commercially available ELISA kit (Quantikine ELISA kit, R&D Systems, Inc., Minneapolis, MN).

**Immunofluorescence staining for terminal deoxynucleotidyl transferase–mediated nick-end labeling, CD31/ErbB1 and CD31/activated caspase-3.** Cryosection sections (8 μm) of DU145 intra-dermal xenografts were fixed with acetone, blocked with 10% goat serum, and incubated with 1:100 dilution of polyclonal anti-ErbB1 and 1:200 dilution of monoclonal anti-CD31 for 2 hours at room temperature. A biotinylated goat anti-rabbit antibody, diluted 1:500, was added for 1 hour; the sections were washed with PBS and incubated with Streptavidin FITC (diluted 1:200, Southern Biotechnology, Birmingham, AL) and a TRITC-conjugated goat anti-rabbit antibody (diluted 1:200, The Jackson Laboratory) for an additional hour. CD31/activated caspase-3 double staining was done using the same protocol described for CD31/ErbB1 staining with the following modifications: the sections were not fixed and an anti-activated caspase-3 antibody was used (diluted 1:10). Terminal deoxynucleotidyl transferase–mediated nick-end labeling staining was done using the In situ Death kit (Roche AG, Basel, Switzerland).

To determine the vessel density, tumor cryosections where stained for CD31 as described above. Pictures encompassing the whole tumor where taken at 10× magnification using a Zeiss Axiosplan microscope. The area of the counted regions was measured using the Openlab 3.1.5 software (Improvision, Lexington, MA). At least three complete tumors were counted per treated group.

**Immunoblot analysis.** Wherever indicated, treatment of cultures with growth factor was done after serum starvation and 2 hours of incubation with the compound. Cells were lysed on ice with buffer containing 1% Triton X-100, 50 mmol/L HEPES (pH 7.5), 150 mmol/L sodium chloride, 5 mmol/L EGTA, 1.5 mmol/L magnesium chloride, 10% glycerol, 0.1 mmol/L sodium orthovanadate, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L sodium pyrophosphate, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Immunoprecipitations were done for 2 hours at 4°C and immunocomplexes were resolved by SDS-PAGE and analyzed by immunoblotting.

**Tumor cell proliferation.** The antiproliferative effect of ZD1839 and PTK787/ZK222584 on EGF-induced proliferation was evaluated via...
bromodeoxyuridine (BrdUrd) incorporation (Biotrak Cell Proliferation ELISA System V.2, Amersham, Buckinghamshire, England). Subconfluent DU145 cells were seeded into 96-well plates at a density of 8 \times 10^3 cells per well and incubated at 37°C and 5% CO_2 in growth medium (MEM EBS containing 10% FCS) for 24 hours. The medium was replaced with MEM EBS containing 0.5% FCS. After 24 hours, the medium was renewed and EGF (50 ng/mL) was added in the presence or absence of ZD1839 or PTK787/ZK222584. Twenty-four hours later, BrdUrd labeling solution was added and BrdUrd incorporation assayed according to manufacturers’ instructions.

**Measurement of vascular endothelial growth factor production by cultured cells.** The concentration of human VEGF in the conditioned medium of DU145 cultures was measured using an ELISA kit (Quantikine ELISA kit, R&D Systems). Cells were plated at the density of 1 \times 10^4 into 96-well plates in growing medium. The medium was replaced and cultures were treated with different concentrations of ZD1839 followed by growth factor stimulation. Twenty-four hours later, quantification of the VEGF in the supernatants and simultaneous quantification of the number of viable cells (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega Corp., Madison, WI) were carried out.

**Endothelial cell proliferation and survival assays.** Subconfluent HUVECs were seeded at a density of 5 \times 10^3 cells per well into 96-well plates coated with 1.5% gelatin and incubated at 37°C and 5% CO_2 in growth medium (PromoCell Nr. C-22110, BioConcept) containing 5% FCS. After 24 hours, the medium was replaced by either basal medium (PromoCell Nr. C-22210, BioConcept) containing 1.5% FCS or growth medium. After 24 hours, the medium was renewed and ZD1839 or vehicle (DMSO) added in the presence or absence of VEGF (20 ng/mL) or EGF (100 ng/mL). Cell proliferation was assessed as described above.

**Fig. 1.** Effect of the combined treatment with PKI166 or ZD1839 and PTK787/ZK222584 on growth of DU145 human prostate tumor xenografts and on plasma VEGF level. DU145 cells (1 \times 10^6) were injected intradermally in the flanks of nude mice. Treatment was started when tumor volume reached \sim 100 mm^3 (28-32 days after inoculation). A, PKI166, PTK787/ZK222584, or the combination of both compounds (50 mg/kg each) were given once a day for two consecutive weeks. B, ZD1839, PTK787/ZK222584, or the combination of both compounds (50 mg/kg each) was given once a day for four consecutive weeks. Each group consisted of 5 to 10 mice. Statistical significance of differences in mean values between treatment groups, compared with the control group, was determined using ANOVA followed by multiple-comparison Dunnett’s test. *, P < 0.05, the level of significance for all tests. Inhibition of tumor growth (T/C) was calculated as described in Materials and Methods in individual animals. Points, means; bars, SE. C, VEGF plasma levels were measured 2 hours after the last dose of the compounds, using a specific ELISA kit, as described in Materials and Methods. Points, means expressed as pg/mL of plasma; bars, SE. *, P < 0.05 versus control (day 8) and ZD1839 (day 0); †, P < 0.05 versus control (day 1) and PTK787 (day 0) by two-way ANOVA and Tukey test.
The effect of ZD1839 on EGF-induced survival of endothelial cells was determined by the Cell Death Detection ELISA Plus Kit (Boehringer Mannheim, Indianapolis, IN). HUVECs were plated as described above. After 24 hours, the medium was replaced by basal medium containing EGF (100 ng/mL) in the presence or absence of ZD1839. After 48 hours, cells were lysed and the supernatants assayed for DNA fragments, as recommended by the manufacturer.

In vitro coculture angiogenesis assay. The effect of ZD1839 and PTK787/ZK222584 on in vitro angiogenesis was assessed as follows. HPASMCs were seeded on 48-well tissue culture plates coated with 100 µL of collagen type I (100 µg/mL in PBS, Sigma) at a density of 2 × 10⁴ cells per well and allowed to attach for 24 hours. The growth medium was then removed and HUVECs (5 × 10⁴ cells per well) were plated on the HPASMCs monolayer. After 24 hours, the medium was replaced with basal medium containing 1.5% FCS in the presence or absence of different growth factors and/or compounds. The medium was renewed at days 1, 3, 5, and 7; at day 9, cells were fixed with 4% PFA for 5 minutes at room temperature. The wells were washed with PBS and 10% normal goat serum, in PBS/0.1% Triton X-100, was added for 30 minutes. Cells were incubated with monoclonal anti-human CD31 in 3% normal goat serum in PBS/0.1% Triton X-100 for 2 hours followed by incubation with biotinylated goat anti-mouse IgG (Southern Biotechnology) in 3% normal goat serum for an additional 2 hours. CD31-positive cells were stained using the Vectastain Elite avidin-biotin complex kit (Vector Laboratories, Burlingame, CA) according to manufacturer’s instructions and detected by 3,3′-diaminobenzidine tetrahydrochloride (Sigma). Cells were imaged at 1.25× magnification using an inverted microscope (Carl Zeiss AG, Munich, Germany) connected to a digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Acquired images were analyzed using Definiens Cellenger software (Definiens AG, Munich, Germany). Identically treated plates of HPASMC were analyzed for human VEGF release as described above.

Statistical analysis. Results are presented as means ± SE. Statistical evaluation of any differences between treatment groups used one-way or two-way ANOVA as appropriate, with post hoc Tukey (pairwise comparisons), or Dunnett (comparisons with controls) used with the ANOVA tests. In some cases, data was transformed to achieve normal distribution. For all tests, the level of significance was set at P < 0.05. Note that with these small group sizes, the desired power level of 0.8 was not always attained. All calculations were done with SigmaStat 2.03.

Results

The in vivo antitumor effect of ErbB and vascular endothelial growth factor receptor kinase inhibitors. We investigated the antitumor activity of the ErbB1 inhibitors PKI166 (Fig. 1A) and ZD1839 (Fig. 1B), either alone or in combination with PTK787/ZK222584, in nude mice bearing DU145 human prostate cancer xenografts. Mice were treated daily with individual drugs or combinations at doses of 50 mg/kg each. Monotherapy with PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A). A similar effect was observed when PTK787/ZK222584 or PKI166 resulted in a reduction of tumor volume of 41% and 44%, respectively, which was not statistically significant. A dramatic cooperative effect, 74% tumor growth inhibition, was observed in mice receiving the combination of both drugs (Fig. 1A).

Fig. 2. Immunofluorescence detection of ErbB1 in DU145 prostate xenografts. Tumor sections were double stained for ErbB1 (green, A) and CD31/PECAM-1 (red, B) and images captured for endothelial cells (CD31, red) were overlapped with images captured for ErbB1; colocalization of the two markers is shown in yellow (CD31/ErbB1, C and D, white arrows).
small cell lung xenograft model and the neuT-driven GeMag orthotopically implanted tumor model (28), evaluating the activity of PTK787/ZK222584 and PKI166, also showed enhanced (possibly synergistic) antitumor effects of combination treatment (Supplementary Table S1). No or nonsignificant loss of body weight was observed in animals treated with combination of agents (data not shown). Analysis of plasma from treated mice showed that whereas the level of released VEGF remained essentially constant over 8 days in vehicle-treated, tumor-bearing mice (P < 0.05, day 1 or 8 versus day 0), treatment with ZD1839 resulted in a time-dependent decrease in the plasma concentrations of VEGF (ZD1839 treated: day 8 versus day 0, P < 0.05; Fig. 1C). In contrast, administration of PTK787/ZK222584 led to a rapid increase in VEGF concentrations, as described previously (ref. 27; PTK787/ZK222584 treated: day 1 versus day 0, P < 0.05), which was higher than that of controls or ZD1839 groups (P < 0.05). This increase in VEGF levels was largely resolved by day 8 (day 8 versus day 0, P > 0.05). Coadministration of ZD1839 and PTK787/ZK222584 blunted the PTK-induced increase in plasma VEGF (PTK + ZD1839; day 1 versus day 0, P > 0.05) and retained the ZD1839 pattern of gradual decline in plasma VEGF levels, although this did not reach statistical significance (Fig. 1C).

We next examined the expression and localization of ErbB1 in sections made from DU145 xenografts. Immunofluorescent staining with an ErbB1-specific antiserum of the untreated (green staining, Fig. 2A) and treated tumors (Supplementary Fig. S1) revealed similarly high receptor levels in tumor cells. Tumor-associated endothelial cells were stained with an anti-CD31 antibody (red staining, Fig. 2B). Double staining of tumor sections with CD31 and ErbB1 antisera revealed that some endothelial cells lining the blood vessels also express ErbB1 (yellow staining, Fig. 2C, a-c; shown enlarged in Fig. 2D, white arrows). Expression of ErbB1 was also observed in the vessels associated with tumors in the PKI166- and PTK787-treated mice, although it was difficult to assess differences in the

Fig. 3. Effect of ZD1839 and PTK787/ZK222584 on in vitro cultured DU145 cells. A, ErbB receptor expression and the EGF- or heregulin (HRG)-induced autophosphorylation was evaluated by immunoblotting. DU145 prostate cancer cells were serum-starved and treated with EGF (50 ng/mL), heregulin (100 ng/mL), or mock treated (−) for 5 minutes. Total cell lysates were immunoprecipitated (IP) with specific anti-ErbB antibodies and immunoblotted with the indicated antibodies. B, quiescent DU145 cells were treated with EGF (50 ng/mL), heregulin (100 ng/mL), or mock treated (−) for 5 minutes. Total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti-P-ERK, anti-ERK, anti-P-AKT, and anti-AKT antibodies. C and D, serum-starved DU145 cells were incubated in the presence or absence of ZD1839 or PTK787/ZK222584 for 1 hour and treated with EGF (50 ng/mL) or mock treated (−) for 5 minutes. Total cell lysates were immunoprecipitated with indicated antibodies. D, total cell lysates were analyzed by immunoblotting with anti-P-ERK and anti-ERK antibodies. E, DU145 cells were incubated with the indicated concentration of ZD1839 or PTK787/ZK222584 in the presence of EGF (50 ng/mL) for 48 hours at 37 °C. Proliferation was assessed by using an in vitro cell proliferation BrdUrd ELSA kit as described in Materials and Methods. Columns, averages of quadruplicate determination; bars, SE. *, P < 0.05 versus control (−); †, P < 0.05 versus EGF (ANOVA and Tukey test). F, after incubation with the indicated concentration of ZD1839 for 24 hours, conditional medium was collected from DU145 cancer cells plated either in serum-free medium and treated with EGF, heregulin, or mock treated (control) or (G) in FCS 10% VEGF secretion was measured using a specific ELSA kit, as described in Materials and Methods and has been normalized for the number of producing cells. Bars, SE. *, P < 0.05 versus control; †, P < 0.05 versus EGF (ANOVA and Tukey or Dunnett’s test).
expression occurred upon treatment due to the collapsed and fragmented appearance of the vessels.

In summary, these results suggest that in the tumor setting, ErbB-specific inhibitors might have direct inhibitory effects, both on tumor cells and associated endothelial cells. Furthermore, because the blockade of tumor growth observed upon treatment with both ErbB1 inhibitors (PKI166 and ZD1839) was accompanied by an inhibition in release of proangiogenic factors (Fig. 1C; data not shown), ErbB receptor inhibition is likely to have indirect antitumor effects. In the following experiments, we examined the in vitro effect of ErbB receptor inhibition on tumor and endothelial cells.

In vitro effect of ZD1839 and PTK787/ZK222584 on DU145 cells. ErbB1, ErbB2, and ErbB3 were detectable in DU145 cells (Fig. 3A). To examine ErbB receptor activity, DU145 cells were treated with EGF or the ErbB3 ligand, heregulin, then cell lysates were prepared and immunoprecipitated (IP) with specific anti-ErbB1 and anti-ErbB2 antibodies and immunoblotted with the indicated antibodies. Total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti-P-ERK and anti-ERK antibodies. Serum-starved HUVEC cultures were incubated in the presence or absence of ZD1839 (0.1 μmol/L) for 1 hour and treated with EGF (100 ng/mL) or mock treated (−) for 15 minutes. Total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti-P-ERK and anti-ERK antibodies. Further, HUVECs were incubated with the indicated concentration of ZD1839 in the absence (Control) or presence of EGF, VEGF, or FCS for 48 hours at 37° C. HUVECs were stimulated with the indicated concentration of VEGF in the absence (Control) or presence of EGF for 48 hours at 37° C. Proliferation was assessed using an in vitro cell proliferation BrdUrd ELISA kit. Columns, averages of quadruplicate determination; bars, SE. *, P < 0.05 versus control; †, P < 0.05 versus EGF (ANOVA and Tukey test, A). ††, P < 0.05 versus control FCS 1% (two-way ANOVA and Tukey test, B). H, quiescent HUVECs were stimulated with the indicated concentration of VEGF, 50 ng/mL of EGF, the combination of the two growth factors or mock treated (−) for 15 minutes. Total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti-P-ERK and anti-ERK antibodies.
concentrations of ZD1839 decreased BrdUrd incorporation to below basal levels, likely due to further inhibition of autocrine activated ErbB1 (Fig. 3E; ref. 37). VEGF did not increase proliferation or survival of DU145 cells (data not shown) and we failed to observe modulation of EGF action upon treatment of the cells with PTK787/ZK222584 (Fig. 3C-E).

The ErbB receptors play a role in the regulation of angiogenesis, via up-regulation of VEGF expression (13, 14, 38, 39). Accordingly, we examined the effect of EGF, herregulin, and ZD1839 on VEGF expression. EGF but not heregulin induced a significant increase (P = 0.003) in VEGF in the conditioned medium of DU145 cultures (Fig. 3F) compared with controls. In the absence of exogenous ligands, ZD1839 showed no significant inhibition of VEGF production. However, starting at 10 nmol/L, ZD1839 significantly impaired the effect of exogenous EGF (P < 0.03). ZD1839 had a minor but inconsistent effect on VEGF production in the presence of herregulin: at 10 (P = 0.003) and 100 (P = 0.045) nmol/L, the VEGF levels were significantly below those in the presence of herregulin (or controls), whereas higher and lower ZD1938 concentrations had no statistically significant effect. ZD1839 treatment also resulted in a significant decrease in VEGF released by cells growing in full serum (Fig. 3G), likely resulting from blockade of the autocrine activated receptor (37). In summary, inhibition of ErbB1 signaling in DU145 tumor cells has a direct negative effect upon tumor cell proliferation and also leads to a decrease in VEGF production. Both of these activities might contribute to the enhanced antitumor activity observed upon addition of ErbB1 inhibitors to PTK787/ZK222584.

Expression and activation of ErbB receptors in endothelial cells. To study the role of ErbB receptors in endothelial cells, we employed two models, HUVECs and human dermal microvascular endothelial cells. ErbB1 and ErbB2 (Fig. 4A) but not ErbB3 and ErbB4 (data not shown) were detected in HUVECs. Addition of the ErbB1 ligands, EGF, heparin-binding EGF, and betacellulin to HUVECs led to an increase in ErbB1 tyrosine phosphorylation and ERK1/2 activation (Fig. 4A and B). None of the ErbB1 ligands induced an increase in ErbB2 phosphorylation (data not shown). In agreement with the data showing that HUVECs do not express heregulin receptors, heregulin treatment had no effect on ERK1/2 activation (Fig. 4B).

Identical results were observed in human dermal microvascular endothelial cells (data not shown) suggesting that in endothelial cells, ErbB1 homodimers have a central role in mediating the response to ErbB family ligands. Addition of 0.1 μmol/L ZD1839 to HUVECs completely abolished EGF-dependent ErbB1 activation (Fig. 4C) and ERK1/2 phosphorylation and resulted in a reproducible slight attenuation of the VEGF-induced ERK1/2 phosphorylation (Fig. 4D). We are currently investigating the specificity of this effect and the possibility of VEGF-mediated ErbB1 transactivation. In contrast, 0.1 μmol/L PTK787/ZK222584 inhibited VEGF-induced but had no effect on EGF-induced ERK1/2 activation.

Active ErbB1 cooperates with vascular endothelial growth factor to promote endothelial cell proliferation. We next examined the consequences of activation or inhibition of ErbB1, on HUVEC proliferation. EGF weakly, and VEGF and FCS more potently, increased BrdUrd incorporation (all P < 0.05, two-way ANOVA; Fig. 4E). Coaddition of ZD1839 (0.01-1 μmol/L) significantly blocked the ability of EGF but not VEGF or serum to stimulate HUVEC proliferation. Treatment with PTK787/ZK222584 reduced VEGF but not EGF-stimulated proliferation (Supplementary Fig. S2).

Considering that in vivo, tumors are likely to be exposed to ErbB family ligands and VEGF, we also examined in vitro proliferation of HUVECs in the presence of EGF and VEGF; 5 and 20 ng/mL VEGF stimulated BrdUrd incorporation, whereas 2 ng/mL was ineffective. In the absence of VEGF, EGF significantly increased BrdUrd uptake and acted in concert with VEGF (at 2 and 5 ng/mL) to promote BrdUrd uptake; no potentiation of VEGF by EGF was seen at 20 ng/mL VEGF (Fig. 4G). With suboptimal concentrations of VEGF, coaddition of EGF also led to an additive increase in ERK1/2 phosphorylation (Fig. 4H). Thus, it is possible that in the presence of physiologically relevant levels of VEGF (293 ± 66 pg/mg of DU145 tumor, n = 9), EGF might play a significant role in modulating tumor angiogenesis.

ErbB1 inhibition induces in vivo apoptosis of tumor cells and associated endothelial cells. The ability of ErbB1 and VEGFR inhibitors to induce apoptosis in vivo was examined next. Terminal deoxynucleotidyl transferase–mediated nick-end labeling staining revealed high levels of apoptotic tumor cells in DU145 xenografts taken from PKI166- treated mice (Fig. 5A). Considering that tumor-associated endothelial cells express ErbB1 (Fig. 2), we assessed the effect of PKI166 treatment on endothelial cell survival. Tumor sections were costained with CD31 antiserum and an antibody recognizing activated caspase-3. This specific early apoptotic marker, stained fewer cells, facilitating identification of costained (yellow) endothelial cells. Tumors taken from vehicle-treated mice lacked endothelial cells undergoing apoptosis (Fig. 5B, g), whereas tumors from PKI166-treated mice displayed activated caspase-3 staining of endothelial cells (Fig. 5B, h). Treatment with PTK787/ ZK222584 caused extensive vessel disorganization and a significant reduction in vessel density (Fig. 5C) within the tumor. Moreover, colocalization could be observed between CD31 and caspase-3 staining (Fig. 5B, i), confirming that inhibition of VEGF signaling promotes endothelial cell apoptosis. Concomitant treatment of mice with PTK787/ZK222584 and PKI166 led to extensive tumor necrosis, rendering it impossible to histologically assess their combined effects.

Inhibition of ErbB1 might induce endothelial cell apoptosis by directly affecting cell survival or, indirectly, by blocking the paracrine secretion of survival factors, such as VEGF. Thus, we examined whether the in vitro antiproliferative effect of ZD1839 on endothelial cells, (Fig. 4F) was accompanied by induction of programmed cell death. Treatment of serum-starved HUVECs with EGF resulted in a concentration-dependent increase in viability, as attested by decreased DNA fragmentation (Fig. 5D). EGF-induced endothelial cell viability was blocked even at the lowest concentration of ZD1839 (0.1 μmol/L) tested. Because EGF does not induce VEGF release from endothelial cells (data not shown), we consider it likely that the endothelial cell apoptosis observed in the presence of the ErbB1 inhibitor in vivo (Fig. 5B) is, at least in part, due to direct effects of the inhibitor on the endothelial cell compartment.

Effect of ZD1839 and PTK787/ZK222584 on endothelial cell sprout formation in a coculture system. The previous results suggested that EGF plays a role in proliferation and survival of endothelial cells, particularly in limiting VEGF concentrations. To investigate whether EGF influences other aspects of endothelial cell biology, we examined its effects in an in vitro
angiogenesis assay consisting of cocultured endothelial and smooth muscle cells. HUVECs when cultured on a monolayer of HPASMCs proliferate and migrate, resulting in the formation of capillary-like sprouts. Cocultures were either left untreated or treated with various peptide ligands in the presence or absence of inhibitors and sprouting was quantified (Fig. 6). Control DMSO-treated HUVECs exhibit a basal level of sprouting (Fig. 6A, a), which was quantified in Fig. 6B (total area) and Fig. 6C (total length). Treatment of the cocultures with VEGF (b), EGF (c), bFGF (d), or PDGF (e) resulted in an increase in sprout number (Fig. 6A), although the image analysis quantification of total sprout area and length failed to show the differences apparent by eye (Fig. 6B-C). PTK787/ZK222584 significantly blocked sprouting in response to VEGF (l), EGF (m), and PDGF (n) and partially blocked the effects of bFGF (p); Fig. 6B-C: VEGF, P = 0.032 and P = 0.042; PDGF, P = 0.002 and P < 0.001; EGF, P = 0.002 and P < 0.001, versus controls within growth factor group in Fig. 6B and C, respectively. To examine the possibility that VEGF exerts a direct effect upon endothelial cells in the coculture assay, whereas EGF, PDGF, and bFGF might act by indirectly stimulating HPASMCs to release VEGF, we used an ELISA assay to measure its release from HPASMCs. The basal level of VEGF released from HPASMCs was increased 2-fold when cultured in the presence of PDGF and EGF and 4-fold following bFGF treatment (all P < 0.001 versus control; Supplementary Fig. S3). PTK787/ZK222584 had no effect upon bFGF- and PDGF-induced VEGF release, suggesting that PTK787/ZK222584’s ability to inhibit the effects of these growth factors is predominantly mediated by blocking endothelial cell VEGFR activity. The lack of complete inhibition of the effects of bFGF by PTK787/ZK222584 is probably due to the documented ability of bFGF to stimulate endothelial cell migration and sprout formation, in a VEGFR-independent fashion (40). PTK787/ZK222584 inhibited EGF-stimulated VEGF production, suggesting that ErbB1 and VEGFR crosstalk may occur (Fig. 4D).

The effects of ZD1839 on sprouting (Fig. 6) and VEGF release (Supplementary Fig. S3) were also examined. In the presence of ZD1839, EGF-stimulated (h) but not VEGF- (g), bFGF- (i), or PDGF-stimulated (j) sprout formation was reduced (Fig. 6B-C; total length of sprouts, P = 0.014 versus control within EGF group). With respect to VEGF release from ZD1839-treated HPASMCs, only EGF-treated cultures showed a significant decrease (Supplementary Fig. S3). These results suggest that the effects of ZD1839 on the coculture assay are, at least partially indirect, resulting from blockade of ErbB1 activity in HPASMCs. Moreover, these experiments highlight ZD1839’s selectivity because it has no effect upon sprouting induced by the other ligands.

ZD1839, and to a greater degree PTK787/ZK222584 (P < 0.05), reduced basal sprouting (Fig. 6A, f and k), without lowering VEGF release from HPASMCs (Supplementary Fig. S3). The PTK787/ZK222584-mediated reduction is likely due to inhibition of the action of HPASMC-released VEGF on endothelial cell VEGFR. The ZD1839-mediated block must occur by alternative mechanisms because HUVECs do not produce VEGF, either basally or in response to EGF, nor do they express VEGF mRNA (data not shown). However, we have detected expression of the ErbB1 ligand hepamin binding-EGF in a gene expression profile analysis (data not shown) raising the possibility that ErbB1 might be activated by an autocrine loop in HUVECs. We propose that this is biologically relevant because ZD1839 blocks basal HUVECs proliferation and survival (Fig. 4F and Fig. 5C) and very likely as a consequence, basal sprouting (Fig. 6A, f). Identical results were observed in human dermal microvascular endothelial cells, suggesting that the EGF effect on sprouting is reproducible in endothelial cells expressing functional ErbB1 (data not shown).

Thus, we propose that ErbB1 signaling provides cues determining changes in endothelial cell morphology in the presence of suboptimal concentrations of VEGF. When ErbB1 is blocked, low levels of VEGF released from the HPASMCs fail to induce sprouting. When VEGF levels are elevated, signals from the VEGFR are sufficient to drive morphologic changes independently from ErbB1 activity (Fig. 6A, g).

Discussion

Aberrant activation of ErbB receptors has been correlated with a more aggressive cancer phenotype and poorer patient prognosis (41, 42). Experimental evidence indicates that ErbB inhibitors can induce tumor stasis or regression and, in addition, increase the antitumor efficacy of conventional and targeted therapies. In particular, there seems a close relationship between the ErbB and VEGFR signaling pathways in progression of solid tumors. The present study was undertaken to assess the effect of selective ErbB inhibitors (PKI166 and ZD1839), given alone or in combination with a VEGFR inhibitor (PTK787/ZK222584; ref. 24). We show that combination therapy exerts a cooperative antitumor and antiangiogenic effect in several experimental tumor models. The DU145 prostate cancer cell line was further used to investigate the mechanisms underlying this cooperativity. The importance of ErbB1 signaling in progression of prostate cancer has been extensively described. Prostate carcinomas and their derived tumor cell lines display autocrine activation of the receptor, due to expression of the ligands EGF and transforming growth factor-α (37, 43, 44) and addition of EGF further stimulates proliferation (45) and invasiveness (46). Conversely, blockade of ErbB1 inhibits growth of cell lines and (47) prostate tumor xenografts (48). We show here that PKI166 treatment caused extensive apoptosis in tumor xenografts and a dose-dependent inhibition of EGF-induced intracellular signaling and cell proliferation in vitro. ErbB1 inhibition abrogated VEGF secretion, both in the in vivo context and in cultured cells in vitro, suggesting that both inhibition of the tumor angiogenic response as well as a direct effect on tumor cell proliferation and survival, underlie the in vivo efficacy of ZD1839 and PKI166 (7, 8, 13). Recently, active ErbB1 has been detected on tumor-associated endothelial cells, an effect ascribed to tumor cell expression of EGF or transforming growth factor-α (9). In the studies presented here, we show that the EGF- and transforming growth factor-α–expressing DU145 cancer cells (37) give rise to tumors associated with ErbB1 expressing endothelial cells. As we show here, in normal culture conditions, both HUVECs and human dermal microvascular endothelial cells express ErbB1. However, we have observed that in vitro treatment of these endothelial cells with EGF-related ligands failed to induce a further increase in ErbB1 mRNA (data not shown), suggesting that alternative mechanisms underlie the increased receptor levels observed in the
endothelium proximal to the ligand expressing tumors (9). Several explanations could be envisaged, an important one being that a localized concentration of a particular ligand could favor the proliferation or migration of endothelial cell clones that express higher levels of its cognate receptor.

We show here that treatment with either an ErbB1 or VEGFR inhibitor resulted in apoptosis of endothelial cells within the prostate tumor. In vitro studies suggested that EGF protects endothelial cells from apoptosis both by directly acting on the endothelial cell compartment as well as via

![Figure 5](image)

**Fig. 5.** Effect of PKI166 and PTK787/ZK222584 on tumor cell and endothelial cell survival. A, representative immunofluorescent images for terminal deoxynucleotide transferase-mediated nick-end labeling (apoptosis) of DU145 xenografts sections from mice treated with vehicle (Control) or PKI166. B, tumor sections were double stained for CD31/PECAM-1 (red) and activated caspase-3 (green) and images captured for endothelial cells (CD31, red) were overlapped with images captured for activated caspase-3 (apoptosis); colocalization of the two markers (yellow). Treatment with PKI166 or PTK787/ZK222584 induced apoptosis of tumor (green) and endothelial cells (yellow). C, tumor sections from mice treated with vehicle (Control), PKI166, or PTK787/ZK222584 were stained for CD31 and the vessel density was determined as described in Materials and Methods; bars, SE. *, \( P < 0.05 \) versus control (ANOVA and Tukey test). D, quiescent HUVECs were treated with the indicated concentration of ZD1839 in the presence of EGF for 48 hours at 37°C. Apoptotic cell death was analyzed using the Cell Death Detection ELISA kit, as described in Materials and Methods. Columns, averages of quadruplicate determination; bars, SE. *, \( P < 0.05 \) versus control; **, \( P < 0.05 \) versus EGF 100 ng/mL (ANOVA and Tukey test).
paracrine induction of survival factors (e.g., VEGF). ErbB1 activation also led to increased endothelial cell proliferation, albeit to a lesser extent than stimulation with VEGF. Notably, the maximal concentration of VEGF used in these in vitro studies was far in excess of that estimated to occur within the tumor environment. When suboptimal, but more physiologically relevant, quantities of VEGF were used, an additive increase in proliferation was observed upon coadministration of EGF. An in vitro angiogenesis assay system was used to show that EGF plays additional roles in endothelial cell biology. Endothelial cells cultured on a smooth muscle cell monolayer undergo morphologic changes resulting in the formation of sprouts. Although sprout formation was observed under basal conditions, increased sprouting occurred upon stimulation with EGF, bFGF, and PDGF. Paracrine production of VEGF by the smooth muscle cell monolayer seemed to underlie the growth factor mediated increase in sprout formation. Notably, inhibition of ErbB1 activity blocked both basal as well as EGF-stimulated sprouting. Sprouting is likely due to proliferation and migration of endothelial cells. Extensive studies failed to show a role for EGF in endothelial cell migration (P. Sini, data not shown) suggesting that the effects of EGF are largely due to promotion of cell division. The effect of ZD1839 on basal sprouting was very similar to the decrease observed upon treatment with PTK787/ZK222584, indicating that both growth factors are required for morphologic changes of endothelial cells under these conditions. In a similar manner, tumors from ErbB1 inhibitor treated mice seemed to contain fewer vessels (data not shown). Although this may be partly a consequence of

Fig. 6. Effect of ZD1839 and PTK787/ZK222584 on sprouting in a coculture system of HUVECs and HPASMCs. A, HUVECs were cocultured with HPASMCs for 9 days in the presence of the indicated growth factors and inhibitors. At days 1, 3, 5, and 7, the culture medium was replaced with fresh medium and at day 9 cells were fixed and immunostained with anti-CD31/PECAM-1 antibody specific for endothelial cells. Bar, 1 mm. CD31-stained HUVECs were analyzed using the Cellmger software.
blocking VEGF production by the tumor, these data suggest that ErbB1 may also have a direct role in tumor angiogenesis. 

In conclusion, we have provided evidence that EGF acts on multiple components of the tumor environment, directly on tumor and endothelial cell proliferation and survival as well as indirectly, via release of proangiogenic factors from tumor and smooth muscle cells. Furthermore, we propose that, in conditions mirroring those found within the tumor, EGF cooperates with VEGF to induce angiogenesis (Supplementary Fig. S4). It is likely that inhibition of the complex crosstalk that occurs between the tumor and endothelial cell compartment underlies the cooperative antitumor effect observed upon coadministration of ErbB1 and VEGFR inhibitors. Our studies suggest that combination treatment with ErbB1 and VEGFR inhibitors may have enhanced beneficial effects in cancer patients compared with treatment with either agent alone.

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References


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