Supplemental data

Materials and Methods

Antibodies and reagents. PTK/ZK was developed as a joint venture between Novartis Pharma and Schering AG. For *in vitro* assays, a 10 mmol/L stock solution of PTK/ZK was prepared in DMSO and diluted in the optimal medium. For *in vivo* studies PTK/ZK was administered in 100% PEG300 (Fluka Chemie AG, Buchs Switzerland). Pab85618, rabbit anti-mVEGF-A (Novartis Pharma) and DC101, rat monoclonal anti-mVEGFR-2 antibody (ImClone Systems, Incorporated, New York, NY) were dissolved in saline solution. For *in vitro* assays, a 0.5 mg/ml stock solution in the optimal medium was prepared. Bevacizumab, monoclonal anti-hVEGF-A (Avastin, Genentech Inc, San Francisco, CA), was diluted in the optimal medium at the concentration of 0.15 mg/mL.

For immunocyto-, immunohisto-chemical and biochemical studies, the following antibodies were used: hamster anti-mCD31 (Chemicon International, Temecula, CA); rat anti-mCD31 (BD PharMingen, San Diego, CA); goat-anti-mVEGFR-2, -anti-mVEGFR-3, -anti-hVEGFR-1, -anti-mLYVE-1, anti-h/mVEGF-B, anti-mVEGF-D (R&D System, Inc, Minneapolis, MN); goat-anti-mVEGFR-1, -anti-h/mVEGF-C (Santa Cruz Biotechnology); rat anti-hVEGFR-2 (RAFL-1) (Ran, S., Huang, X., Downes, A. and Thorpe, P.E. Neoplasia 5: 297-307, 2003); flag-tag anti-hVEGFR3 (AF8, K. Ballmer, PSI, Villigen, Switzerland); rabbit-anti-mLYVE-1 (clone SZ 2017), -anti-hPodoplanin (clone SZ 1787) (Novartis Pharma); anti-ERK1/2, anti-P-EKR1/2, anti-AKT, anti-P-AKT (Cell Signaling Technology, Beverly, MA); goat anti-rabbit ALEXA 568, goat anti-rat ALEXA 546, donkey anti-goat ALEXA 555 or 488, goat anti-hamster ALEXA 488 secondary antibodies (Molecular Probes); anti-Flag (Sigma, St. Louis, MO); anti-rat

FITC-conjugated, anti-rabbit TRITC-conjugated, donkey anti-goat biotin SP-conjugated secondary antibodies (Jackson Laboratory, West Grove, PA); streptavidin ALEXA 568 (Molecular Probes). Recombinant human VEGF-C (R&D System, Inc., Minneapolis, MN) and recombinant dog VEGF-A (Novartis Pharma) were used. The concentrations of mouse and human VEGFs in the conditioned medium of tumor cells were measured using an ELISA kit as previously described (28).

Immunocyto- and histo-chemical analysis. Immunocytochemical analysis was performed as described (28). Briefly cryosections (8 μm) of B16 lymph node metastases were blocked in horse serum (HS) and stained with anti-mVEGFR-1 (4 μg/ml), anti-mVEGFR-2 (10 μg/ml) or anti-mVEGFR-3 (2 μg/ml). Secondary antibodies used were donkey anti-goat ALEXA 555 (1:1000) or donkey anti-goat biotin SP-conjugated (1:500) followed by streptavidin ALEXA 568 (1:1000). The sections were washed, incubated with either hamster anti-mCD31 (1:100) or goat anti-mLYVE-1, followed by goat anti-hamster ALEXA 488 or donkey anti-goat ALEXA 488 (1:1000). For CD31/LYVE-1 double staining cryosections were blocked in normal goat serum (NGS), incubated with rat anti-mCD31 (1:200) and rabbit anti-mLYVE-1 (1:100) overnight at 4°C. The sections were washed and incubated with anti-rat and anti-rabbit secondary antibodies (1:200, Jackson Laboratory). For immunocytochemistry, cells were fixed 10 min in 4% paraformaldehyde, slides were rinsed with PBS and preincubated 30 min with blocking buffer (0.1% Triton X-100, 10% goat or horse serum in PBS). Slides were then incubated 2 hrs with antibodies to: hVEGFR-1 (10 μg/ml), hVEGFR-2 (1:100), hVEGFR-3 (1:25), hPodoplanin (1:100), mVEGFR-1 (4 μg/ml), mVEGFR-2 (10 μg/ml)

and mVEGFR-3 (2 μg/ml) in PBS plus 3% NGS, 0.1% Triton X-100. After washing, either goat anti-rat ALEXA 546 or donkey anti-goat ALEXA 555 were added for 1 hr. Nuclei were counterstained with Hoechst 33342 (1:4000).

Fluorescence-activated cell sorting. LECs, MB-MDA435, A375 and A549 cells were trypsinized, Fc-blocked with PBS/0.5 % BSA supplemented with 1μg of mouse IgG/10^5 cells for 15 min. Anti-VEGFRs antibodies (monoclonal anti-hVEGFRs phycoerythrin (PE)-conjugated, R&D System) were added and staining performed according to manufacturers' instruction. Podoplanin-antibody stained cells were washed in PBS/0.5% BSA and PE-conjugated anti-rabbit (1:100, BD PharMingen) was added. B16/BL6 cells were harvested and Fc-blocked with PBS/0.5% BSA supplemented with 5% NGS in the absence or presence of 0.3% Triton, then incubated with goat anti-mVEGFR-1 (R&D System) or PE-conjugated rat anti-mVEGFR-2 antibody (BD PharMingen) for 1h at 4°C. Cells stained for VEGFR-1 were then incubated with anti-goat secondary antibody FITC-conjugated. Staining of the cells with an isotype IgG was performed as negative control. Cells were washed and suspended in 500 μl of PBS/0.5% BSA and analyzed with a FACSCalibur using Cell Quest Software (Becton Dickinson).

Figure legends

Figure 1

Effects of PTK/ZK, DC101 and Pab85618 on primary tumor growth and lymph node metastases of the B16/BL6 mouse melanoma model and on the *in vivo* angiogenesis chamber model. *A, B & C left panels,* B16/BL6 primary tumor growth was measured at days 7, 14, and 21 after tumor cell inoculation; inhibitors were given at the indicated doses starting at day 7. *A, B & C middle panels,* average weight of cervical lymph node metastases measured on day 21 following treatment with the indicated inhibitor between day 7 and 21. *A, B & C right panels,* on the fourth day after implantation, animals were sacrificed, the chamber was removed and weighed. Points and columns, means; bars, ±SE. *, $P < 0.05$ treatment groups versus control group (one-way ANOVA and post hoc Holm-Sidak test).

Figure 2

Effect of PTK/ZK or Bevacizumab on *in vitro* cultured human lymphatic endothelial cells (LECs). *A,* immunofluorescent and flow cytometry detection of VEGFRs and Podoplanin in LECs. *B,* quiescent LECs were incubated 1 hour with 1000 nM PTK/ZK or vehicle control and then treated with either VEGF-C or VEGF-A or mock treated (-) for 15 minutes. VEGFR-3 immunoprecipitates were immunoblotted with the indicated antibodies. *C,* quiescent LECs were incubated 1 hour with 1000 nM PTK/ZK, 100 ng/ml Bevacizumab or vehicle control and then treated with either VEGF-C, VEGF-A or mock

treated (-) for 15 minutes. Total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti P-AKT, P-ERK, AKT and ERK antibodies. *D,* quiescent LECs were incubated with the indicated concentration of PTK/ZK or Bevacizumab in the absence (control) or presence of VEGF-A or VEGF-C for 48 hours at 37 ºC. Cell death was measured using the YO-PRO assay as described in the *Material and Methods*. *E,* quiescent LECs were incubated with the indicated concentration of PTK/ZK or Bevacizumab in the absence (control) or presence of VEGF-A or VEGF-C for 48 hours at 37 ºC. Proliferation was assessed using an *in vitro* cell proliferation BrdUrd ELISA kit. Columns, mean; bars, SE. *, $P < 0.05$ versus control; º $P < 0.05$ versus VEGF-A; # $P < 0.05$ versus VEGF-C (ANOVA and Holm-Sidak or Tukey test).

Figure 3

PTK/ZK does not sensitize tumor cells to 5-FU-based chemotherapy. *A, B & C,* Cultures of the indicated growing tumor cell lines were incubated in the absence or presence of PTK/ZK (1000 nM) combined with increasing concentrations of 5-FU (0-10 μM) for 24 hours at 37 ºC. Cell death and cell number were measured using the YO-PRO assay as described in the *Material and Methods*. Columns, mean; bars, SE. *, $P < 0.01$ versus 5-FU 0 μg/ml (One-Way ANOVA and Holm-Sidak test).

Figure 4

Expression of VEGFs in tumor cell lines. *A & B* Conditioned medium was collected from MB-MDA435 and A375 cells plated in either basal medium or growth medium. VEGF-A and VEGF-C secretion was measured using an ELISA kit as described in *Materials and*

Methods. Total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti-VEGF-B.