PTK787/ZK 222584, a Novel and Potent Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases, Impairs Vascular Endothelial Growth Factor-induced Responses and Tumor Growth after Oral Administration


ABSTRACT

PTK787/ZK 222584 (1-[4-chloroanilino]-4-[4-pyridylmethyl] phthalazine succinate) is a potent inhibitor of vascular endothelial growth factor (VEGF) receptor tyrosine kinases, active in the submicromolar range. It also inhibits other class III kinases, such as the platelet-derived growth factor (PDGF) receptor β, tyrosine kinase, c-Kit, and c-Fms, but at higher concentrations. It is not active against kinases from other receptor families, such as epidermal growth factor receptor, fibroblast growth factor receptor-1, c-Met, and Tie-2, or intracellular kinases, such as c-Src, c-Abl, and protein kinase C-α. PTK787/ZK 222584 inhibits VEGF-induced autophosphorylation of kinase insert domain-containing receptor (KDR), endothelial cell proliferation, migration, and survival in the nanomolar range in cell-based assays. In concentrations up to 1 μM, PTK787/ZK 222584 does not have any cytotoxic or antiproliferative effect on cells that do not express VEGF receptors. After oral dosing (50 mg/kg) to mice, plasma concentrations of PTK787/ZK 222584 remain above 1 μM for more than 8 h. PTK787/ZK 222584 induces dose-dependent inhibition of VEGF and PDGF-induced angiogenesis in a growth factor inhibitor model, as well as a tumor cell-driven angiogenesis model after once-daily oral dosing (25–100 mg/kg). In the same dose range, it also inhibits the growth of several human carcinomas, grown s.c. in nude mice, as well as a murine renal carcinoma and its metastases in a syngeneic, orthotopic model. Histological examination of tumors revealed inhibition of microvessel formation in the interior of the tumor. PTK787/ZK 222584 is very well tolerated and does not impair wound healing. It also does not have any significant effects on circulating blood cells or bone marrow leukocytes as a single agent or impair hematopoietic recovery after concomitant cytotoxic anti-cancer agent challenge. This novel compound has therapeutic potential for the treatment of solid tumors and other diseases where angiogenesis plays an important role.

INTRODUCTION

Angiogenesis, the formation of new vessels from an existing vascular network, is an essential event in a variety of physiological and pathological processes. Under physiological conditions, angiogenesis is restricted to processes such as embryogenesis, ovulation, and wound healing. Angiogenesis also occurs in pathological processes such as inflammation (1–4), rheumatoid arthritis (5–8), ocular neovascularization (9–13), psoriasis (14–16), tumor growth, and the formation of metastases (17, 18).

Of the numerous growth factors and cytokines that have been shown to have angiogenic effects, VEGF2 appears to be a key factor in pathological situations that involve neovascularization as well as enhanced vascular permeability (19, 20). The VEGF receptors, Flt-1 (VEGF-R1; Ref. 21) and KDR (VEGF-R2; Ref. 22), are almost exclusively located on endothelial cells (23). Expression of these receptors is low in normal tissues and only up-regulated during the development of these pathological states when neovascularization occurs (24, 25). Both receptors have seven immunoglobulin-like domains in their extracellular region, a single transmembrane-spanning domain, and an intracellular split tyrosine kinase domain and belong to the same family of receptors as PDGFR, c-Kit (a receptor for stem cell factor), c-Fms (a receptor for colony-stimulating factor), Flt-3, and Flt-4. Flt-1 binds VEGF-A and VEGF-B (26, 27) and the related placenta growth factor (28, 29), whereas KDR binds VEGF-C and VEGF-D (30, 31) in addition to VEGF-A. VEGF-C and VEGF-D are both ligands and activators of Flt-4 (VEGF-R3), which is expressed on the endothelial cells of lymphatic vessels (32–34). Although activation of Flt-1 was shown to mediate biological responses, such as endothelial and monocyte cell migration and tissue factor induction (27, 28, 35–38), in cells expressing only Flt-1 or in Flt-1-deficient cells transfected with Flt-1 cDNA, stimulation with VEGF induces only weak receptor phosphorylation and no significant mitogenic response (37, 38). In contrast to Flt-1, KDR is strongly autophosphorylated upon VEGF stimulation and mediates a mitogenic response (39, 40).

Gene knock-out experiments for VEGF (41, 42) as well as its receptors (43–46) have highlighted the pivotal role of the VEGF/VEGF receptor system in the development of the embryonic vascular system. Various different approaches have been used to interfere with the VEGF/VEGF receptor system in adult animals and thereby determine the role of VEGF receptors in the various pathological states. These approaches include VEGF neutralizing antibodies (47–49), antibodies against the VEGF receptors (50, 51), recombinant soluble VEGF receptor proteins (52, 53), a tetracycline-regulated VEGF expression system (54, 55), and dominant-negative mutants of the VEGF receptors (56). Results from these approaches suggest the VEGF/VEGF receptor system is a novel and attractive therapeutic target for suppression of pathological neovascularization and, in particular, for inhibiting tumor growth (57, 58).

Our aim was to design a low molecular weight synthetic molecule

2 The abbreviations used are: VEGF, vascular endothelial growth factor; Flt, fms-like tyrosine kinase; KDR, kinase insert domain-containing receptor; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PTK787/ZK 222584, 1-[4-chloroanilino]-4-[4-pyridylmethyl] phthalazine succinate; HUVEC, human umbilical cord endothelial cells; CHO, Chinese hamster ovarian cells; GST, glutathione S-transferase; PKC, protein kinase C; BrdUrd, 5-bromo-2-deoxyuridine; bFGF, basic fibroblast growth factor; Cd2c, cell cycle-dependent kinase 2; Flik, fetal liver kinase; Fms, feline myeloosarcoma; mAb, monoclonal antibody; Tie, tyrosine kinase with immunoglobulin.

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In accordance with
that potently and selectively blocks the VEGF/VEGF receptor system after oral administration, suitable for the chronic therapy of VEGF-dependent pathological neovascularization. In this report, we describe the pharmacological profile of a potent inhibitor of the VEGF tyrosine kinases that fulfills this goal. Although other synthetic molecules have been reported that inhibit the VEGF receptor kinases (59, 60), to our knowledge this is one of the first low molecular weight inhibitors of the VEGF/VEGF receptor system reported to be active at inhibiting VEGF-mediated processes after oral administration (61).

MATERIALS AND METHODS

**Substances.** PTK787/ZK 222584 (62) and SU5416 (60) were synthesized in the Department of Oncology Research (Novartis Pharmaceuticals). PTK787/ZK 222584 was discovered and was profiled in collaboration with the Institute of Molecular Medicine (Tumor Biology Center), as well as the Oncology Research Laboratories of Schering AG. The studies described in this report were performed with either a dihydrochloride or succinate salt. For **in vitro** assays, a stock solution of 10 mM PTK787/ZK 222584 was prepared in DMSO. This was diluted further in buffer or medium so that the concentration of DMSO in assay systems did not exceed 0.01%. For **in vivo** studies, the vehicle for the dihydrochloride salt was distilled water. The succinate salt was suspended in vehicle containing 5% DMSO and 1% Tween 80 in distilled water. The VEGF used in all **in vitro** and **in vivo** assays was human VEGF_165 produced in Escherichia coli (63). The HUVECs were obtained from PromoCell (Heidelberg, Germany). The KDR-transfected CHO cells were obtained from the Institute of Molecular Medicine (Tumor Biology Center). The tumor cell lines were obtained from American Type Culture Collection (Rockville, MD), except for CWR-22, which was obtained from Dr. Pretlow (Case Western Reserve University School of Medicine, Cleveland, OH).

**VEGF Receptor Tyrosine Kinase Assays.** The **in vitro** kinase assays were performed in 96-well plates as a filter binding assay, using the recombinant GST-fused kinase domains expressed in baculovirus and purified over glutathione-Sepharose. γ-[^32]P]ATP was used as the phosphate donor, and poly-(Glu:Tyr 4:1) peptide was used as the acceptor. Recombinant GST-fusion proteins were diluted in 20 mM Tris-HCl buffer (pH 7.5) containing 1–3 mM MnCl₂, 3–10 mM MgCl₂, 0.25 mM/mg polyethylene glycol 20000, and 1 mM DTT, according to their specific activity. Each GST-fused kinase was incubated under optimized buffer conditions [20 mM Tris-HCl buffer (pH 7.5), 1–3 mM MnCl₂, 3–10 mM MgCl₂, 3–8 μM poly-(Glu:Tyr 4:1), 0.25 mM/mg polyethylene glycol 20000, 8 μM ATP, 10 μM sodium vanadate, 1 mM DTT, and 0.2 μCi [γ-[^32]P]ATP in a total volume of 30 μl in the presence or absence of a test substance for 10 min at ambient temperature. The reaction was stopped by adding 10 μl of 250 mM EDTA. Using a 96-well filter system, half the volume (20 μl) was transferred onto a Immobilon-polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was washed by a constant concentration of VEGF (50 ng/ml), bFGF (0.5 ng/ml), or FCS (5%), in the presence or absence of PTK787/ZK 22254. As a control, wells without growth factor were also included. After 24 h of incubation, BrdUrd labeling solution was added, and cells incubated an additional 24 h before fixation, blocking, and addition of peroxidaselabeled anti-BrdUrd antibody. Bound antibody was then detected using 3.3',5'-tetramethylbenzidine substrate, which results in a colored reaction product that is quantified spectrophotometrically at 450 nm.

**Endothelial Cell Survival Assay.** To determine whether PTK787/ZK 222584 could block the endothelial cell survival properties of VEGF, the effects of this compound on cell survival in the presence and absence of VEGF was determined under serum-free conditions. HUVECs (1 × 10⁵ cells/well) were cultured in fibronectin-coated wells (eight-well chamber slides) in the absence of serum with or without VEGF (10–100 ng/ml) and in the presence of increasing concentrations of PTK787/ZK 222584. After 48 h, slides were fixed in cold 70% ethanol, and RNase A digested (200 μg/ml) before the DNA was labeled with saturating concentrations of propidium iodide (25 μg/ml) in Coplin jars. Slides were oversprayed with glyc erin-PBS containing propidium iodide at the same propidium iodide concentration. Analysis of the cells was performed using a Laser Scanning Cytometer (Compucyte Corporation, Cambridge, MA). The argon ion laser 488-nm excitation (5 mW) and a 570-nm long pass filter using the ×20 objective were used to collect propidium iodide fluorescence. This was used as the contouring and thresholding parameter for collecting data on cells remaining in the culture wells at the end of the experiment. Scan area was set to encompass the available culture area for each of the chambers. Data acquisition/analysis parameters were set to include only single cells and to exclude debris and aggregates.

**Endothelial Cell Migration Assay.** As a test of the ability of this compound to inhibit another functional response to VEGF that is important for angiogenesis, an endothelial cell migration assay was used. Plates (24-well) were coated with 1.5% gelatin and fitted with circular fences as a barrier to prevent cells from growing in the center of the well. Subconfluent HUVECs were seeded into the outer area (1 × 10⁴ cells/well) and then incubated at 37°C and 5% CO₂ in growth medium. After 24 h, the fences were removed, and the growth medium was replaced by basal medium containing human VEGF (10 ng/ml), in the presence or absence of PTK787/ZK 222584. To inhibit the cell proliferation, 50 μg/ml 5-fluorouracil (Roche, Basel, Switzerland) was added. After 72 h of incubation, the cells were fixed and stained with Diff-Quik (Dade Behring AG, Düdingen, Switzerland), and the number of migrated cells was counted under a binocular microscope, using the software KS-400 (Carl Zeiss Jena, Jena, Germany). The number of cells in the wells with VEGF or serum and vehicle alone was taken as 100% (control), and changes in cell number in wells with different concentrations of PTK787/ZK 222584 were calculated as a percentage of the control values.

**Assays of Antiproliferative Activity against Cells Not Expressing the VEGF Receptors.** Potential antiproliferative effects of PTK787/ZK 222584 were tested using cells that do not express the VEGF receptors, the human tumor cell lines A431 (epithelial carcinoma) and DU145 (prostate carcinoma). Cells were seeded into 96-well microtiter plates (1.5 × 10⁴ cells/well) and incubated overnight. PTK787/ZK 222584 was added in serial dilutions on day 1. The plates were then incubated for 6 days. After incubation, the cells were fixed with 3.3% glutaraldehyde, washed with water, and stained with 0.05% methylene blue. After washing, the dye was eluted with 3% HCl, and the absorbance measured with a SpectraMax 340 microtiter plates reader at 665 nm.
Vessel Sprout Formation Assay. The ability of PTK787/ZK 222584 to inhibit angiogenesis was tested in an \textit{in vitro} model of capillary sprout formation (64). Fragments (1 mm$^3$) of rat aorta were imbedded in a fibrin gel (500 μl) in 24-well plates and incubated with medium containing 10% FCS, in the presence or absence of increasing concentrations of PTK787/ZK 222584. After 6 days, capillary density was quantified from images viewed under an inverse microscope using a computerized imaging system (KS-400, Carl Zeiss Jena, Jena, Germany).

Determination of Plasma Concentrations after Oral Dosing in Mice. The aim was to develop a compound that would inhibit VEGF-induced angiogenesis after oral administration. We tested whether PTK787/ZK 222584 is absorbed after oral administration in female mice (MAG). Plasma concentrations of free base were also measured after single oral administration of 50 mg/kg of the dihydrochloride or succinate salt. Both salts were formulated in 5% DMSO/1% Tween 80. As a reference, plasma concentrations of SU5416 were measured after oral administration at the same dose and with the same vehicle. Oral dosing was done by gavage, and the mice had free access to food and water during the experiment. At the allotted times, four mice were sacrificed from each treatment group, and heart blood was collected into heparinized tubes. Plasma samples were analyzed immediately for the free base of the compounds by reversed-phase high-pressure liquid chromatography. The plasma samples were deproteinized by the addition of an equal volume of acetonitrile, followed by thorough mixing and centrifugation. The supernatant was analyzed directly. A standard curve was constructed from plasma spiked with known concentrations of compound and processed and analyzed as described above. Concentrations down to 0.1 μM (the lowest concentration in the standard curve) could be determined.

\textbf{In Vivo Growth Factor-induced Angiogenesis Model.} To determine whether PTK787/ZK 222584 inhibits VEGF-mediated angiogenesis \textit{in vivo}, we tested the effects of PTK787/ZK 222584 on the angiogenic response induced by VEGF in a growth factor implant model in mice. To test the specificity of the response, the effects on PDGF-induced angiogenesis were also tested. A porous Teflon chamber (volume, 0.5 ml) was filled with 0.8% w/v agar containing heparin (20 units/ml) with or without growth factor (3 μg/ml human VEGF, 2 μg/ml human PDGF) that was implanted s.c. on the dorsal flank of C57/C6 mice. The mice were treated with PTK787/ZK 222584 (12.5, 25 or 50 mg/kg dihydrochloride p.o. once daily) or vehicle (water) starting 1 day before implantation of the chamber and continuing for 5 days after. At the end of the treatment, the mice were killed, and the chambers were removed. The vascularized tissue growing around the chamber was carefully removed and weighed, and the blood content was assessed by measuring the hemoglobin content of the tissue (Drabkins method; Sigma, Deisenhofen, Germany). We have shown previously that these growth factors induce dose-dependent increases in weight and blood content of the tissue growing (characterized histologically to contain fibroblasts and small blood vessels) around the chambers and that this response is blocked by antibodies that specifically neutralize the growth factors (65).

\textbf{In Vivo Tumor Cell-induced Angiogenesis Model.} To determine whether PTK787/ZK 222584 inhibits angiogenic response mediated by tumor cells \textit{in vivo}, we tested the effects of PTK787/ZK 222584 on the angiogenic response induced by epithelial carcinoma A431 (2 × 10$^6$) encapsulated in alginate beads and implanted s.c. on the dorsal flank of nude mice as described in detail previously (66). The mice were treated with PTK787/ZK 222584 (50 mg/kg dihydrochloride p.o. once daily) or vehicle starting on the day of implantation of the beads and continuing for 12 days after. On day 12, the mice were killed 20 min after injection of 0.1 ml of FITC-labeled high molecular weight dextran solution (M, 150,000; 100 μg/kg). The blood content of the alginate beads was quantified by measuring the uptake of fluorescent dextran into the alginate implant, as described previously (66).

\textbf{Nude Mouse Human Tumor Xenograft Model.} To determine the effects of PTK787/ZK 222584 on tumor growth, its effects were tested against various human tumors grown s.c. in nude mice. Tumor growth was initiated by s.c. injection of the human carcinoma cell lines (10$^6$ cells; A431 epithelial carcinoma, LS174T colon carcinoma, and HT-29 colon carcinoma) or by transplantation of tumor fragments (PC-3 prostate carcinoma, DU145 prostate carcinoma, and CWR-22 prostate carcinoma; 25 mg) from carrier mice. Drug treatments were initiated when tumor volumes of 25–100 mm$^3$ were attained. PTK787/ZK 222584 was given in doses from 25 to 100 mg/kg p.o., once or twice daily. Tumor growth was monitored weekly by measuring perpendicular diameters. Tumor areas were determined as the product of the largest diameter (a) and its perpendicular (b) according to the formula [tumor area = a × b], and tumor volumes were calculated from the determination of the largest diameter (a) and its perpendicular (b) according to the formula [tumor volume = a × b$^2$/2].

\textbf{Tumor Histology.} To determine whether tumor growth inhibition by PTK787/ZK 222584 was associated with inhibition of tumor vessel formation, groups of mice bearing the A431 epithelial carcinoma produced from tumor fragments were used for histological examination. The tumor-bearing mice were treated with either PTK787/ZK 222584 (50 mg/kg dihydrochloride p.o.) or vehicle. Two animals from each group (n = 6) were killed each week, i.e., 7, 14, and 22 days after starting treatment (14, 21, and 29 days after tumor transplantation), for histological examination of the tumor. The tumor tissue was quickly frozen in isopentane at −130°C and stored at −70°C. For the visualization of the blood vessel endothelial cells, cryosections were stained with anti-CD31 antibody (rat anti mouse; Pharmingen, San Diego, CA; dilution, 1:5000) using dianaminobenzidine as the chromagen (ABC-Vectastain kit; Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin.

\textbf{Syngeneic Mouse Model.} To determine whether PTK787/ZK 222584 inhibits tumor growth and metastasis formation in immune competent mice, its effects were also tested in the orthotopic murine renal carcinoma model (RENCA). Tumors were initiated by injection of 1 × 10$^5$ cells into the subcapsular space of the kidney. Drug treatment was initiated 1 day after tumor cell inoculation. Mice received either PTK787/ZK 222584 (50 mg/kg dihydrochloride p.o. once daily) or vehicle (distilled water). Three weeks after starting therapy, mice were killed for determination of metastasis formation in the lungs and regional lymph nodes in the abdominal cavity.

\textbf{Wound Healing Model.} Full-thickness linear incisional wounds (3 cm) were made along the dorsal midline of young male Sprague Dawley rats (250 g; n = 10 group) and cauterized with mattress sutures. Rats were administered PTK787/ZK 222584 (5, 20, or 50 mg/kg dihydrochloride i.p., once per day, days −2 to 7) or 1 ml/kg saline i.p. (once per day, days −2 to 7). PTK787/ZK 222584 was dosed i.p. rather than p.o. to avoid handling stress to the wound. The 50-mg/kg i.p. dose was estimated to be equivalent to a 100-mg/kg p.o. dose by pharmacokinetic evaluation. As a control of impaired wound healing, another group of animals received a daily dose of dexamethasone (5 mg/kg, i.m.) starting 1 day before the incision was made. Rats were sacrificed, skin sections (30 × 8-mm strips) within the wounds were excised, and the tensile strength of the healed wound (measured by stretching the wound to the breaking point with a Universal Tensile Strength Machine, model 144501; Zwick, Ulm, Germany) was measured. The skin sections were fixed (4% paraformaldehyde in PBS pH 7.2), embedded, sectioned, and stained with H&E for qualitative histological assessment.

\textbf{Evaluation of Effects on Circulating and Bone Marrow Leukocytes.} To determine the effects of PTK787/ZK 222584 on hematopoiesis, normal bone marrow was exposed to 50 mg/kg PTK787/ZK 222584 or vehicle, p.o. once per day for 21 days. To determine the effect of PTK787/ZK 222584 on hematopoietic recovery after cytotoxic insult, BALB/c mice (n = 5/group) were treated with 100 mg/kg cyclophosphamide or saline (i.p., days 3, 5, and 7) to reduce the total number of bone marrow cells and circulating blood cells. The mice were also treated with 50 mg/kg PTK787/ZK 222584 or vehicle (p.o., once per day). After 21 days of treatment, the mice were sacrificed, and a blood sample was taken by puncture of the vena cava and using EDTA anticoagulant. The tibifibula were exposed by dissection and removed by cutting the bone as near to the malleolus and condyle as possible. The fibula was cut off, and the bone fragment was weighed. The bone marrow was obtained by inserting a 23-gauge needle into the condyle end of the bone, and the cells were flushed out with 1 ml of PBS containing 1 unit/ml heparin. The resulting “plug” of cells could be homogeneously suspended by gentle vortexing. The leucocytes, erythrocytes, and platelets were enumerated by use of a Sysmex TOA E-5000 blood cell counter (Digitana AG, Horen, Switzerland).

\textbf{RESULTS}  

\textbf{Inhibition of Protein Kinases by PTK787/ZK 222584.} PTK787/ZK 222584 inhibited all of the VEGF receptor tyrosine kinases tested. It was slightly more potent against KDR (VEGF-R2)
than against Flt-1 (VEGF-R1) and even less potent against the mouse homologue of the human KDR, Flk-1 (Table 1). It also inhibited Flt-4 (VEGF-R3) at even higher concentrations (Table 1). PTK787/ZK 222584 was also active against other tyrosine kinases belonging to the same family of tyrosine kinase receptors as the VEGF receptors, i.e., the PDGFR-β, c-Kit, and c-Fms (Table 1). SU5146 was less selective than PTK787/ZK 222584 for KDR; SU5416 inhibited c-Kit, and KDR was in the same concentration range, with Flt-1 at even lower concentrations. It inhibited PDGFR-β with similar potency to PTK787/ZK 222584.

PTK787/ZK 222584 did not inhibit kinases from other enzyme families in concentrations up to 10 μM. The various kinases tested include FGF-R1, c-Met, Tie-2, epidermal growth factor receptor, c-Src, v-Abl, PKC-α, and Cdc2.

Effects of PTK787/ZK 222584 on VEGF-induced Cellular Responses. To determine whether PTK787/ZK 222584 is taken up by cells to reach its intracellular target, its effects on receptor phosphorylation were tested in cell-based assays. Measurement of VEGF-induced autophosphorylation of KDR in a double antibody chemiluminescence assay, using either HUVECs or CHO cells transfected with the KDR receptor, showed that PTK787/ZK 222584 inhibits the VEGF-induced phosphorylation with an IC₅₀ of 17 ± 2 nm (n = 4) and 34 ± 2 nm (n = 14) for the HUVECs (Fig. 1) and CHO cells, respectively. SU5416 stimulated autophosphorylation of KDR in both cell types under these assay conditions.

Table 1 Inhibitory activity of PTK787/ZK 222584 against class III receptor tyrosine kinases

<table>
<thead>
<tr>
<th>Kinase</th>
<th>PTK787/ZK 222584 IC₅₀ (nm)</th>
<th>SU 5416 IC₅₀ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF receptor/KDR</td>
<td>0.037 ± 0.002, n = 20</td>
<td>0.20 ± 0.03, n = 3</td>
</tr>
<tr>
<td>VEGF receptor/Flt-1</td>
<td>0.077 ± 0.012, n = 8</td>
<td>0.008 ± 0.007</td>
</tr>
<tr>
<td>VEGF receptor/Flk</td>
<td>0.27 ± 0.04, n = 6</td>
<td>0.78 ± 0.06, n = 3</td>
</tr>
<tr>
<td>Flt-4</td>
<td>0.66 ± 0.62, n = 2</td>
<td>ND</td>
</tr>
<tr>
<td>c-Kit</td>
<td>0.73 ± 0.05, n = 8</td>
<td>0.47 ± 0.41</td>
</tr>
<tr>
<td>c-Fms</td>
<td>1.4 ± 0.1, n = 9</td>
<td>ND</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>0.58 ± 0.08, n = 8</td>
<td>0.68 ± 0.35</td>
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* Values are mean ± SE. ND, not determined.

To test the effects of PTK787/ZK 222584 on functional responses to VEGF, its effects on HUVEC proliferation, migration, and survival were tested. PTK787/ZK 222584 inhibited thymidine incorporation induced by VEGF in HUVECs with and IC₅₀ of 7.1 ± 5.1 nm (n = 4). SU5416 inhibited VEGF-induced thymidine incorporation in HUVECs with an IC₅₀ of 16 ± 4 nm (n = 4). In concentrations up to 1 μM, neither PTK787/ZK 222584 nor SU5416 inhibited the response to bFGF or serum.

Analysis of the HUVECs labeled with propidium iodide by laser scanning cytometry showed that VEGF prevented death and loss of cells under serum deprivation (Fig. 2). PTK787/ZK 222584 dose dependently inhibited VEGF-induced survival of endothelial cells in the same dose range as it inhibited endothelial cell proliferation (Fig. 2). When viewed under a fluorescence microscope, condensed and fragmented nuclei typical of cells undergoing apoptosis could be clearly seen in the serum-deprived cells in the absence of VEGF. They were not seen in the samples treated with VEGF but were induced by
increasing concentrations of PTK787/ZK 222584. Apoptosis was also shown by the leftward shift of the DNA histograms in Fig. 2 with 1–10 nM PTK787/ZK 222584. With higher concentrations, most of the cells had died and were lost during preparation of the slides. In a cell migration assay, PTK787/ZK 222584 also inhibited VEGF-induced HUVEC migration dose dependently (IC50, 58 ± 10 nM; n = 8).

A selective VEGF inhibitor should not inhibit the proliferation of cells that do not express the VEGF receptors in the same concentration range as it inhibits VEGF receptor-mediated processes. This was tested using the human epithelial carcinoma cell line A431 and the human prostate carcinoma DU145. PTK787/ZK 222584 had no effects on the proliferation of A431 and DU145 in concentrations up to 1 nM. At very high concentrations (1000-fold higher than required to inhibit KDR phosphorylation in the cell-based assays), PTK787/ZK 222584 inhibited tumor cell proliferation (IC50; 17 ± 1 μM and 18 ± 2 μM for A431 (n = 4) and DU145 (n = 5), respectively).

To test its effects on capillary formation, the effects of PTK787/ZK 222584 were tested on the formation of sprouts from pieces of rat aorta embedded in a fibrin gel. PTK787/ZK 222584 inhibited sprout formation dose dependently with an IC50 of 675 ± 64 nM (n = 8; Fig. 3).

Plasma Concentrations of PTK787/ZK 222584 after Oral Administration to Mice. To determine whether PTK787/ZK 222584 is absorbed, plasma concentrations were measured after oral administration. Peak concentrations of free base reached about 30 μM 30 min after oral administration of either salt form. At 8 h after administration, the concentrations were still >1 μM (Fig. 4). After oral administration of SU5416 at the same dose as PTK787/ZK 222584, plasma concentrations were barely detectable (Fig. 4) and fell below the level of detection after 2 h.

Effects of PTK787/ZK 222584 on VEGF-induced Angiogenesis in a Growth Factor Implant Model in Vivo. To determine whether PTK787/ZK 222584 could inhibit a VEGF-mediated response in vivo after oral administration, its effects were tested in a growth factor implant model in mice. With this model, VEGF and PDGF induce

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The angiogenic response mediated by tumor cells in vivo, we tested the effects of PTK787/ZK 222584 on the angiogenic response induced by the human epithelial carcinoma A431 encapsulated in alginate beads implanted s.c. in mice. PTK787/ZK 222584 inhibited the tumor cell-induced angiogenic response (Fig. 6).

Effects of PTK787/ZK 222584 on the Growth of Several Human Tumors in Nude Mice. The effects of PTK787/ZK 222584 were investigated on the growth of a range of human tumors grafted s.c. in the nude mouse. All of these tumors produce VEGF when grown as cells in culture or as s.c. xenografts in nude mice. (data not shown). PTK787/ZK 222584 induced dose-dependent inhibition of growth of several of these human tumors after daily oral administration in doses between 25 and 100 mg/kg (Table 2; Figs. 7 and 8). The most responsive tumor type of those investigated was prostate. With the DU145, carcinoma tumor growth was inhibited by up to 70%. With the CWR-22 (Fig. 8), no macroscopically visible tumors were found when the mice were sacrificed after 80 days in three of seven of the PTK787/ZK 222584-treated mice. With all other tumors, the maximum effect was to slow tumor growth (Table 2; Fig. 7). The maximum effects were obtained with daily dosing of about 50–100 mg/kg.

Effects of PTK787/ZK 222584 on Tumor Vascularization. Histological examination of the human epithelial carcinoma A431, grown as s.c. xenografts in the nude mouse, revealed that microvessels had begun to form in the interior of the tumor mass 14 days after implantation of tumor fragments (Fig. 9A). With time, tumor size increased with growth of larger vessels at the interface between the tumor and host tissue but no change in microvessels in the interior of the tumor (Fig. 9, A, C, and E) PTK787/ZK 222584 in an oral dose of 50 mg/kg once daily from day 7 after tumor implantation slowed tumor growth (Fig. 7). This was associated with a reduced occurrence of microvessels in the interior of the tumor mass with time and the appearance of necrosis in the tumor center (Fig. 9, B, D, and F). PTK787/ZK 222584 had no effect on larger established vessels, particularly those at the edge of the tumor (Fig. 9, B, D, and F).

Effects of PTK787/ZK 222584 on Tumor Growth and the Development of Metastasis in a Mouse Syngeneic Orthotopic Tumor Model. The effects of PTK787/ZK 222584 on primary tumor growth, as well as the development of metastases, were investigated in immune competent mice. For this purpose, an orthotopic model of murine renal cell carcinoma (RENCA) was used. PTK787/ZK 222584 inhibited the growth of the primary tumor (T/C) (mean increase of tumor volumes of treated animals divided by the mean increase of tumor volumes of control animals multiplied by 100); 34%) and also resulted in substantial reduction in the number of metastases in the lymph nodes and lungs (T/C, 29 and 65%, respectively; n = 12 for vehicle group and n = 9 for the treated group; P < 0.05). Histological examination of blood vessels stained with CD 31 in the primary tumor in the kidney and in the lung metastasis also revealed an inhibition of microvessel formation (T/C for microvessel density, 45 and 42%, respectively).

PTK787/ZK 222584 was well tolerated in all of the in vivo experiments and had no significant effects on body weight, general wellbeing of the animals, or macroscopic effects on any body organs at the time of sacrifice.

Effects of PTK787/ZK 222584 on Wound Healing. Because angiogenesis is a critical part of wound healing, we tested for the possible impairment of wound healing attributable to inhibition of VEGF signaling. PTK787/ZK 222584, administered in doses ranges of 5, 20, or 50 mg/kg i.p. once daily did not impair the healing of a full-thickness incisional wound in rats nor alter its tensile strength (Fig. 10). In contrast, in rats treated with dexamethasone (5 mg/kg i.m. once daily), the wound was slower to heal than in vehicle-treated rats, and the tensile strength was significantly reduced by ~40%. Furthermore, the wounds from dexamethasone-treated rats had a lower de-

Table 2: Effects of PTK787/ZK 222584 on the growth of s.c. implanted human carcinomas in nude mice

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Salt/Dose/Schedule</th>
<th>Effect*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-431 epidermoid carcinoma</td>
<td>Succinate/25, 50, and 75 mg/kg p.o./once daily</td>
<td>Significant inhibition of tumor growth at 50 and 75 mg/kg doses (maximum T/C, 40%; Fig. 7)</td>
</tr>
<tr>
<td>LS174T colon carcinoma</td>
<td>Succinate/25, 50, and 75 mg/kg p.o./once daily</td>
<td>Significant inhibition of tumor growth at 50 and 75 mg/kg doses (maximum T/C, 40%)</td>
</tr>
<tr>
<td>HT-29 colon carcinoma</td>
<td>Succinate/25, 50, and 75 mg/kg p.o./once daily</td>
<td>Significant inhibition of tumor growth at 25, 50, and 75 mg/kg doses (maximum T/C, 60%)</td>
</tr>
<tr>
<td>PC-3 prostate carcinoma</td>
<td>Dihydrochloride/50 mg/kg p.o./once daily</td>
<td>Significant inhibition of tumor growth (maximum T/C, 50%)</td>
</tr>
<tr>
<td>DU145 prostate carcinoma</td>
<td>Succinate/25 and 50 mg/kg p.o./once or twice daily; succinate/100 mg/kg p.o./once daily</td>
<td>Significant inhibition of tumor growth at all doses and schedules (maximum T/C, 33%)</td>
</tr>
<tr>
<td>CWR-22 prostate carcinoma</td>
<td>Succinate/50 mg/kg p.o./once daily</td>
<td>Significant inhibition of tumor growth (stable disease or cures; Fig. 8)</td>
</tr>
</tbody>
</table>

* T/C% (mean increase of tumor volumes of treated animals divided by the mean increase of tumor volumes of control animals multiplied by 100); n = 10/treatment group.
grecy of cellularity, as evidenced by a marked decrease in the influx of mononuclear phagocytes and fibroblasts and a subsequent decrease in newly formed matrix in the wound biopsies (data not shown). This was not seen in wounds from PTK787/ZK 222584-treated rats.

Effects of PTK787/ZK 222584 on Circulating and Bone Marrow Leukocytes. Given the effectiveness of PTK787/ZK 222584 in inhibiting c-Kit, the potential of PTK787/ZK 222584 to disrupt hematopoiesis was evaluated. After 21 days of PTK787/ZK 222584 treatment (50 mg/kg p.o., once daily), the circulating blood cells of BALB/c mice (10.6 ± 1.5 x 10^8 leukocytes/μl; 8.7 ± 0.23 x 10^8 erythrocytes/μl; 867 ± 64 x 10^6 platelets/μl) and bone marrow leukocytes (log 8.39 ± 0.04 cells/g bone) were not statistically different from those of vehicle-treated controls (9.4 ± 0.6 x 10^8 leukocytes/μl; 8.5 ± 0.5 x 10^8 erythrocytes/μl; 732 ± 31 x 10^6 platelets/μl and log 8.23 ± 0.03 leukocytes/g bone). Cyclophosphamide treatment significantly reduced both circulating leukocytes and bone marrow leukocytes (Fig. 11), as well as circulating erythrocytes and platelets (not shown), but the mice were able to recover by 8–14 days after the last dose. When administered in combination with cyclophosphamide, 50 mg/kg PTK787/ZK 222584 (p.o., once per day) did not enhance the depletion of, nor impair the recovery of, the blood or bone marrow leukocytes (Fig. 11) nor erythrocytes or platelets (not shown). A higher dose of 100 mg/kg p.o. of PTK787/ZK 222584 administered only in the recovery phase after the cyclophosphamide challenge also had no significant effects on recovery of any of these parameters.

DISCUSSION

In this report, we describe the pharmacological profile of a potent and p.o. bioavailable inhibitor of VEGF-mediated responses, based on the molecular mechanism of inhibition of the VEGF receptor tyrosine kinases. This is one of the first synthetic inhibitors of the VEGF receptor tyrosine kinases that is so active after oral dosing. In enzymatic assays using recombinant protein kinases, PTK787/ZK 222584 has been shown to be a potent inhibitor of the VEGF receptor tyrosine kinases. It is most potent against KDR, the human VEGF-R2, and exhibits slightly weaker inhibition of Flt-1, the human VEGF-R1, and even weaker inhibition of Flk-1, the mouse homologue of KDR, and Flt-4, the receptor found in the lymphatic system. At similar and higher concentrations, PTK787/ZK 222584 also inhibits other kinases belonging to the same class as the VEGF receptors, i.e., PDGFR-β, c-Kit, and c-Fms. Under the same assay conditions, the reference compound, SU5416, also inhibits these class III kinases and shows even less selectivity for KDR. Protein kinases tested that belong to other families, such as EGF-R, c-Abl, c-Src, PKC-α, and Cdc2, are not inhibited by PTK787/ZK 222584. It is also inactive against FGF-R1, c-Met, and Tie-2, other receptors implicated to play a role in angiogenic processes.

We have shown that PTK787/ZK 222584 is active against KDR also in cellular VEGF-induced receptor phosphorylation assays, using endothelial cells that naturally express KDR, or a transfected cell line. In these cellular assays, PTK787/ZK 222584 is active in the 20 nM range, indicating that it penetrates into cells and reaches its intracellular target. We have also demonstrated that PTK787/ZK 222584 selectively inhibits VEGF-mediated cellular responses in the same dose range; PTK787/ZK 222584 inhibits VEGF-mediated cell proliferation, cell survival, and cell migration. In the same concentration range, PTK787/ZK 222584 does not inhibit proliferation of cells that do not express the VEGF receptors.

As evidence that PTK787/ZK 222584 can lead to a disruption of neovascularization, we have shown that it inhibits capillary-like sprout formation in an in vitro angiogenesis assay. In addition, we have shown that PTK787/ZK 222584 inhibits VEGF-induced vascularization in an growth factor implant model and a tumor cell-driven angiogenesis model in vivo. Moreover, histological examination of tumors used in the studies described in this study, as well as additional studies in other tumor models (67), have shown that treatment of tumors in animal models can significantly reduce tumor vascularization.
As well as inhibiting tumor vascularization, PTK787/ZK 222584 inhibits ischemia-induced retinal neovascularization in newborn mice (68). PTK787/ZK 222584 not only inhibits VEGF-mediated neovascularization but also VEGF- and tumor-induced increases in vessel permeability (67).

In contrast to all other reported inhibitors of VEGF (antisense,
antibodies and the previously reported receptor tyrosine kinase inhibitor, SU 5416) and many other angiogenesis inhibitors with other mechanisms of action, PTK787/ZK 222584 is well absorbed after oral administration, with plasma concentrations remaining well above concentrations required to inhibit VEGF-induced receptor phosphorylation for 8 h in mice. This pharmacokinetic profile indicates that PTK787/ZK 222584 can be given p.o. Indeed, we were able to demonstrate that VEGF-mediated responses can be effectively blocked after once daily oral dosing with PTK787/ZK 222584. In a growth factor implant angiogenesis model in which VEGF induces concentration-dependent growth of vascularized tissue, PTK787/ZK 222584 dose dependently blocked the increase in vascularized tissue. It also blocked a similar but weaker response to PDGF, in a higher dose range, consistent with its weaker activity against the PDGFR-β tyrosine kinase in the in vitro assays. After oral dosing, PTK787/ZK 222584 also inhibited the angiogenic response induced by the human epithelial tumor cell (A431) in a s.c. implant in mice. The growth of s.c. tumors arising from this tumor cell line was also inhibited after oral dosing in nude mice, and this was associated with a reduction in the number of microvessels in the interior of the tumor.

PTK787/ZK 222584 inhibited tumor growth in several different tumor models. The best effects were seen against the prostate tumors. All of these tumors are very slow growing. The growth of the CWR-22, a very slow growing tumor, was completely inhibited in some mice. The growth of all of the other tumors investigated in the nude mouse was only inhibited or slowed, and there was no tumor regression observed. The effective dose range of PTK787/ZK 222584 in the various in vivo models was between 50 and 100 mg/kg daily. It has to be considered that PTK787/ZK 222584 is ~7-fold less potent against the mouse receptor tyrosine kinase Flk than the equivalent human kinase, KDR. Therefore, the doses needed in patients may be lower than can be directly extrapolated from the mouse models.

Histological studies with the A431 tumor as well as the RENCA tumor after 1–3 weeks of treatment showed decreases in microvessels in the tumor. Further studies in other tumor models have also revealed that PTK787/ZK 222584 significantly reduces the number of microvessels in the tumor (67). The disappearance of the capillaries under treatment is consistent with our observation that PTK787/ZK 222584 inhibits VEGF-induced survival of endothelial cells in vitro.

Not all vessels in the tumors were affected by treatment with PTK787/ZK 222584, particularly larger vessels at the periphery of the tumor. This indicates that only newly forming capillaries are inhibited, and more stable and mature vessels formed before treatment is initiated, or induced by other angiogenic factors, are not sensitive to VEGF inhibition. More mature, larger vessels consisting of more than
a single layer of endothelial cells may be resistant to treatment with VEGF inhibitors. The residual tumor growth seen with many of the s.c. tumors in nude mice models appears to be driven by the remaining the larger vessels, particularly at the periphery of the tumor, that are not reduced by treatment with PTK787/ZK 222584.

Our findings that PTK787/ZK 222584 is less effective against some tumors than others is consistent with studies reported using the tyrosine kinase inhibitor, SU 5416 (60), and neutralizing antibodies against VEGF (47–49). Although we and others have shown that almost all tumor cell lines in vitro and tumors grown in vivo produce VEGF, cytokines or growth factors other than VEGF may also contribute to endothelial cell survival and tumor angiogenesis and may even be up-regulated when the effects of VEGF are inhibited. In vitro we have observed that PTK787/ZK 222584 does not induce apoptosis of endothelial cells if any serum is present in the medium. This suggests that there are several different factors supporting endothelial survival and tumor vascularization. Anti-VEGF therapy may be more effective against some types of tumors than others, and future therapy may necessitate a combination of antiangiogenic agents with different mechanisms of action, as well as conventional therapies targeting the tumor cell.

Inhibition of tumor growth was not just confined to tumors growing s.c.; PTK787/ZK 222584 also inhibited primary tumor growth and the growth of metastases in an orthotopic tumor models in immune competent mice. The effect on the metastasis was even greater than the effects on the primary tumor. Inhibition of metastasis formation might be attributable to both the decreased vascularization of a primary tumor, leading to reduced escape routes for metastatic cells, as well as the decreased vascularization of metastasis restricting their growth. PTK787/ZK 222584 also inhibits VEGFR-3, the receptor expressed in the lymphatic system, and this activity might also contribute to its antimetastatic effects.

Because both VEGF and PDGF have been implicated to play a role in wound healing, a surprising finding was that there was no impairment of wound healing in any of the tumor models requiring surgery or impairment in healing or the strength of an incisional wound in a rat wound healing model. Either there is a redundancy of angiogenic factors, or VEGF and PDGF do not play a vital role in the wound healing process. This study suggests that PTK787/ZK 222584 may not impair the normal wound healing process and may be safe to give to patients after surgery. It can be speculated that the larger vessels forming at the periphery of the implanted tumors in nude mice may actually represent a wound healing response, rather than tumor-induced angiogenesis. This may explain why these vessels are not inhibited by PTK787/ZK 222584 in the nude mice and warrants further investigation.

PTK787/ZK 222584 as a single agent had no significant effects on circulating blood cells or bone marrow leukocytes and did not impair hematopoietic recovery after a cytotoxic anticancer agent challenge. This is also quite surprising considering the inhibitory effects of PTK787/ZK 222584 on KDR and c-Kit, class III kinases that are expressed on hematopoietic cells. This suggests a redundancy of hematopoietic growth factors and also indicates that PTK787/ZK 222584 will not enhance the hematopoietic toxicity of cytotoxic agents.

In all of the in vivo experiments described in this report and in further studies that have been performed (67, 68) as well as in 4-week Good Laboratory Practice toxicology studies in rats and dogs, PTK787/ZK 222584 was extremely well tolerated with no effects on body weight or animal behavior, and no target organ toxicity was observed. Taken together, all of these observations indicate that PTK787/ZK 222584 is well tolerated after at least 1 month of chronic therapy and is much better tolerated than conventional antitumor therapies. It remains to be explored whether chronic therapy as a single agent or cyclic therapy in combination with conventional antitumor therapies will be the best approach for the treatment of cancer.

PTK787/ZK 222584, a synthetic, low molecular weight, p.o. bioavailable, and well-tolerated molecule, offers many advantages over angiogenesis inhibitors reported previously. Several molecules with different mechanisms of action have been reported, some of which have entered clinical trials. We have shown that PTK787/ZK 222584 is as active and much better tolerated than TNP-470 (69, 70) in several of our tumor models as well as having the advantage of oral dosing. Although angiostatin (71) and endostatin (72) were reported originally to be more effective inhibitors of tumor growth than we report here for PTK787/ZK 222584, not all laboratories have been able to reproduce the same degree of in vitro and in vivo efficacy that was reported originally. Moreover, both molecules are difficult to manufacture in large quantities and are not p.o. active. An additional attractive feature of compounds that inhibit the effects of VEGF is that VEGF is not only an angiogenic factor but also a potent vascular permeability factor. This may give VEGF inhibitors additional therapeutic applications over antiangiogenic agents with other mechanisms of action. Soluble VEGF receptors (52, 53) and antibodies against VEGF (47–49) or its receptors (50, 51) have also been proposed as agents to block VEGF, but compared with PTK787/ZK 222584, have the disadvantages of large proteins (difficult to manufacture, cost, immunogenic potential, and need for parenteral administration). Other VEGF receptor tyrosine kinase inhibitors, such SU5416, have also been reported (59, 60), but we have shown that this compound has poor oral bioavailability and is even less specific for the VEGF receptor tyrosine kinases than PTK787/ZK 222584. Moreover, another VEGF receptor tyrosine kinase inhibitor, SU 6668, has been reported that is p.o. active, but it is even less specific, inhibiting bFGF as well as the class III kinases. This might lead to greater antitumor efficacy but also to more side effects.

Our data show that PTK787/ZK 222584 is a potent, p.o. active, and well-tolerated inhibitor of VEGF-mediated responses. The excellent oral activity and tolerability of this compound favor its use for prolonged treatment, not only for cancers dependent on VEGF for their vascularization, but also for other diseases where VEGF-mediated angiogenesis plays a key role in the pathogenesis.

REFERENCES

3. Amano, S., Rohan, R., Karoki, M., Tolentino, M., and Adams, A. P. Requirement for VEGF, cytokines or growth factors other than VEGF may also contribute to its antimetastatic effects.


ORALLY ACTIVE INHIBITOR OF VEGF RECEPTOR TYROSINE KINASES


PTK787/ZK 222584, a Novel and Potent Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases, Impairs Vascular Endothelial Growth Factor-induced Responses and Tumor Growth after Oral Administration


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