Pharmacological Characterization of CP-547,632, a Novel Vascular Endothelial Growth Factor Receptor-2 Tyrosine Kinase Inhibitor for Cancer Therapy


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ABSTRACT

Signaling through vascular endothelial growth factor (VEGF) receptors (VEGFRs) is a key pathway initiating endothelial cell proliferation and migration resulting in angiogenesis, a requirement for human tumor growth and metastasis. Abrogation of signaling through VEGF by a variety of approaches has been demonstrated to inhibit angiogenesis and tumor growth. Small molecule inhibitors of VEGF tyrosine kinase have been shown to inhibit angiogenesis, inhibit tumor growth, and prevent metastases. Our goal was to discover and characterize an p.o. active VEGF-2 small molecule inhibitor. A novel isoaxazole, CP-547,632, was identified as a potent inhibitor of the VEGF-2 and basic fibroblast growth factor (FGF) kinases (IC_{50} = 11 and 9 ns, respectively). It is selective relative to epidermal growth factor receptor, platelet-derived growth factor β, and other related TKs. It also inhibits VEGF-stimulated autophosphorylation of VEGF-2 in a whole cell assay with an IC_{50} value of 6 ns. After oral administration of CP-547,632 to mice bearing NIH3T3/H-ras tumors, VEGFR-2 phosphorylation in tumors was inhibited in a dose-dependent fashion (EC_{50} = 590 ng/ml). These plasma concentrations correlated well with the observed concentrations of the compound necessary to inhibit VEGF-induced corneal angiogenesis in BALB/c mice. A sponge angiogenesis assay was used to directly compare the inhibitory activities of CP-547,632 against FGF receptor 2 or VEGFR-2; this compound potently inhibits both basic FGF and VEGF-induced angiogenesis in vivo. The antitumor efficacy of this agent was evaluated after once daily p.o. administration to athymic mice bearing human xenografts and resulted in as much as 85% tumor growth inhibition. CP-547,632 is a well-tolerated, orally-bioavailable inhibitor presently under clinical investigation for the treatment of human malignancies.

INTRODUCTION

Cancer is the second leading cause of death in the United States. This year 554,000 people are expected to die of cancer in the United States, whereas 1.3 million are expected to be diagnosed with cancer (American Cancer Society).1 Therapies that provide lasting remissions represent the greatest unmet medical need in oncology. Agents with improved side effect profiles and improved efficacy in refractory or metastatic disease would provide substantial improvement to existing therapies. The molecular revolution has led to an increased understanding of the pathways responsible for oncogenesis and tumor growth. We are just beginning to reap the fruits of these labors by understanding of the pathways responsible for oncogenesis and tumor growth and metastasis. The molecular revolution has led to an increased understanding of the pathways responsible for oncogenesis and tumor growth. We are just beginning to reap the fruits of these labors by understanding of the pathways responsible for oncogenesis and tumor growth and metastasis. Our goal was to discover and characterize an p.o. active VEGF-2 small molecule inhibitor. A novel isoaxazole, CP-547,632, was identified as a potent inhibitor of the VEGF-2 and basic fibroblast growth factor (FGF) kinases (IC_{50} = 11 and 9 ns, respectively). It is selective relative to epidermal growth factor receptor, platelet-derived growth factor β, and other related TKs. It also inhibits VEGF-stimulated autophosphorylation of VEGF-2 in a whole cell assay with an IC_{50} value of 6 ns. After oral administration of CP-547,632 to mice bearing NIH3T3/H-ras tumors, VEGFR-2 phosphorylation in tumors was inhibited in a dose-dependent fashion (EC_{50} = 590 ng/ml). These plasma concentrations correlated well with the observed concentrations of the compound necessary to inhibit VEGF-induced corneal angiogenesis in BALB/c mice. A sponge angiogenesis assay was used to directly compare the inhibitory activities of CP-547,632 against FGF receptor 2 or VEGFR-2; this compound potently inhibits both basic FGF and VEGF-induced angiogenesis in vivo. The antitumor efficacy of this agent was evaluated after once daily p.o. administration to athymic mice bearing human xenografts and resulted in as much as 85% tumor growth inhibition. CP-547,632 is a well-tolerated, orally-bioavailable inhibitor presently under clinical investigation for the treatment of human malignancies.

1 This article is dedicated to the memory of Dr. Edward A. Glazer, a member of the Pfizer team and a driving force in the discovery of CP-547,632, who passed away in 2002.

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The abbreviations used are: FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; FBS, fetal bovine serum; Flk-1, fetal liver kinase receptor 1 (murine vascular endothelial growth factor receptor 2); HDLP, homologous dialysis liquid perfusate; HUVEC, human umbilical vein endothelial cell; MVD, microvessel density; PY, phosphotyrosine; TK, tyrosine kinase; TMB, 3,3',5,5'-tetramethylbenzidine dihydrochloride; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; IP, immunoprecipitation; FGFR: qd, once daily.

The in-growth of host microvasculature into a solid tumor, referred to as angiogenesis, is now widely appreciated to be a necessary event for tumor growth beyond a few mm^3 in volume (1, 2). Therefore, it is expected that drugs that block the molecular events responsible for tumor angiogenesis will be effective against a broad spectrum of tumor types. Angiogenesis is a highly regulated process, and although essential for embryogenesis, this process is restricted in adults to ovulation, cyclical endometrial proliferation, and wound repair (3). Therefore, inhibitors of angiogenesis are predicted to be better tolerated than conventional cytotoxic cancer therapies that affect all rapidly growing cells. Because the target is the invading normal host vasculature, another potential attribute of an antiangiogenesis approach may be the avoidance of drug resistance commonly associated with conventional anticancer modalities, which target genetically unstable tumor cells (4). Moreover, with p.o. or i.v. delivery, neovascular endothelial cells will be the first cell types encountered by extravasating drugs, providing the possibility that tumor penetration by these agents may not be necessary. Angiogenesis is the hallmark of many vascular proliferative disorders other than cancer, including psoriasis, rheumatoid arthritis, diabetic retinopathy, and age-related macular degeneration (5–7). Therefore, it is reasonable to expect that well-tolerated antiangiogenesis agents will be efficacious in these disease states as well.

Angiogenesis is a complex biological process that relies on a variety of growth factors and signaling cascades to stimulate the migration and proliferation of the component cell types and to establish functional blood vessels. Many factors have been shown to affect endothelial proliferation resulting in angiogenesis in vivo, such as angiopoietins, FGFs (especially bFGF, PDGF, platelet-derived endothelial cell growth factor, EGF, angiogenin, and VEGF; Refs. 8–13). Of these factors, research has focused on VEGF, attributable in part to its endothelial cell specificity, and its temporal and spatial expression at times of physiological and pathological blood vessel growth (14). VEGF was originally identified as a tumor-secreted vascular permeability factor (15), subsequently shown to be secreted by many other cell types, including macrophages and folliculostellate cells (16). VEGF is regulated by many physiological conditions commonly associated with tumors and ischemic tissues, such as hypoxia and hypoglycemia (17). There are two high-affinity receptors for VEGF that appear to be exclusively expressed and functional on endothelial cells, fms-like TK receptor 1 (also VEGFR-1) and Flk-1 (also kinase domain receptor or VEGFR-2; Refs. 18, 19). Like VEGF, the expression of these receptors is spatially and temporally regulated on endothelium in times of vasculogenesis and angiogenesis, and is up-regulated in response to hypoxia and hypoglycemia (20, 21). The binding of VEGF to its receptors induces receptor dimerization and
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After the assay was carried out as above, and IC₅₀ values were calculated as percentage inhibition of control. Selectivity assays were performed as described above using pure recombinant enzyme (generated as described above, except for TK with immunoglobulin and epidermal growth factor homology domains 2, which is an EGFR chimera) and ATP concentrations that approximated the Kᵅ for the enzyme.

Cell-Based Phospho-VEGFR-2 Inhibition Assay. Porcine aortic endothelial cells stably expressing full-length VEGFR-2 have been described previously (30). Cells were seeded at 1.6 × 10⁵ cells/ml in 2-ml growth medium (Ham's F-12 medium supplemented with 10% FBS, 50,000 units each penicillin and streptomycin, and 500 μg/ml gentamicin) per well in six-well plates. On day 2, the growth medium was replaced with serum-depleted medium (as above, but with 0.1% FBS and 0.1% BSA), and cells were incubated overnight. Immediately before compound addition, the medium was replaced with serum-depleted medium without BSA. Compounds were diluted in 100% DMSO, added to the cells at a final DMSO concentration of 0.25% v/v, and incubated at 37°C for 1 h. The cells were then stimulated with 500 ng/ml VEGF (Becton Dickinson, prepared in serum-depleted medium supplemented with 10 μM NaVO₄) and incubated as above for an additional 8 min. The medium was removed and the cells washed once with PBS supplemented with 1 μM NaVO₄, then lysed with 1 ml of immunoprecipitation assay buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 25 mM EDTA, 1% NP40, 0.25% sodium deoxycholate, 2 μM NaVO₄, and 1 EDTA-free protease inhibitor tablet per 25 ml]. Cell lysates were centrifuged at 14,000 rpm to pellet cellular debris, transferred to a new tube containing 4 μg anti-Flk-1 (Santa Cruz Biotechnology Laboratories; C20), and incubated with agitation overnight at 4°C. The antibody-protein complex was captured with protein A agarose beads (Santa Cruz Biotechnology Laboratories) for 30 min at 4°C and the protein boiled off in the presence of DTT. After electrophoresis and transfer to Immobilon-P membranes, the blots were probed with antibodies recognizing either the protein (monoclonal anti-Flk-1; Santa Cruz Biotechnology Laboratories; A3) or anti-PY-HRP. After incubation of the blot in enhanced chemiluminescence reagent (Amerham), bands were visualized on film or using the Lumi-ImagerF1 (Roche).

HUVEC Mitogenesis. HUVECs were obtained from Clonetics and grown according to the manufacturer's instructions. Cells (10⁴ cells/ml) were seeded on cover slips coated with collagen, 500 μg/ml poly-glu-tyr. The media was replaced with 1 ml of growth medium containing 0.2% DMSO and 10 ng/ml recombinant human VEGF (Becton Dickinson). After 2 h, the growth medium was replaced with 1 ml of serum-depleted medium. Compounds were added 24 h later (diluted in DMSO to a final concentration of 0.2% v/v). Cells were then stimulated with VEGF or FGF (10 ng/well), diluted in HBSS. After 3 hrs, 1 μCi [³H]thymidine was added to each well, and after 8 h, cells were collected and the incorporation of [³H]thymidine was determined by the incorporation of [³H]thymidine in the cells detected in a scintillation counter.

Animals for in Vivo Studies. Female athymic mice were injected with 1 × 10⁶ NIH-3T3/H-Ras cells (NIH3T3 cells transfected with full-length activated H-ras oncogene) on day 1. When tumors were ~300 mm³, the mice received...
compound or vehicle (5% Gelucire 44/14 in sterile water, Gattefosse, Saint-Priest, France) p.o. One to 3 h after dosing, the tumors were excised and frozen in liquid N\textsubscript{2}. For the pharmacokinetic/pharmacodynamic analysis, the tumors were removed at the various times postdose. The tumors were homogenized in 1 ml of lysis buffer per 200 mg tumor [lysis buffer: 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 1 mM EDTA, 1% Glycerol, 1% Triton X-100, 1.6 mM Na\textsubscript{3}VO\textsubscript{4}, 10 mM NaF, 25 mg/liter Soy Bean Trypsin Inhibitor, and EDTA-free complete Protease Inhibitor Tablets]. The tumors were homogenized using a dounce homogenizer mounted on a standing drill press. Homogenates were spun for 5 min at 14,000 rpm, and the supernatant was aliquoted to 96-well polypropylene plates or tubes on dry ice. Total protein concentration was determined using BCA protein assay (Pierce). Protein A coated plates (96-well; Pierce) were blocked with 100 l/well cold blocking buffer (Tris-buffered saline, 0.1% Tween 20, and 3% BSA) for 60 min on a plate shaker at room temperature. The blocking buffer was replaced with 0.5 g anti-Flk-1 in 100 l cold blocking buffer per well and incubated for 60 min, room temperature, on a plate shaker. The plates were washed with Tris-buffered saline and 0.1% Tween 20 using a Skatron Plate washer. Tumor lysate (100 l diluted to 5 mg/ml total protein in lysis buffer without protease inhibitors) was added to the plate and incubated for 3 h, room temperature, on a plate shaker. The plates were washed as above, then incubated with 15 ng of HRP-PY54 per well (in blocking buffer) for 30 min at room temperature. The plates were washed as above and PY quantitated using TMB as described above.

Plate IP and Western Blot Analysis. Tumor lysates were incubated on protein A plates as described for the ex vivo ELISA. The plates were then washed as above. We added 45 l 3X Laemmli loading buffer to the wells, and the plate was placed on a plate shaker for 5 min with vigorous shaking. The plate was heated 8 X 30 s in a microwave oven. After each 30-s heating the plate was removed and tapped gently to cover the wells with the loading buffer. Complete heating was determined by the color change in the loading buffer. The plate was then shaken vigorously on a plate shaker for 5 min. The samples were loaded onto Novex NuPAGE 4–12% Bis-Tris Gels, and electrophoresis was carried out with 3-[N-morpholino]propanesulfonic acid buffer running buffer with antioxidant added to the inner chamber. Proteins were transferred to polyvinylidene fluoride membranes in Tris-Glycine Transfer Buffer with 10% methanol. PY levels were detected using anti-PY-HP diluted in blocking buffer. VEGFR-2 levels were detected using anti-VEGFR-2 antibody followed by antirabbit HRP reporter antibody. Enhanced chemiluminescence (Amerham) was used according to the manufacturer’s instructions. The VEGFR-2 band was quantitated using the MasterScan Densitometer.

**Sponge Angiogenesis Assay.** Surgical sponges were cut into 5 X 5 mm squares and soaked with 1 l of either bFGF or VEGF (Becton Dickinson) per

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**Table 1. Kinase, and cell potency and selectivity**

<table>
<thead>
<tr>
<th>Kinase (IC\textsubscript{50}, nM)</th>
<th>Cell proliferation (IC\textsubscript{50}, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR-2 11</td>
<td>14</td>
</tr>
<tr>
<td>bFGF 9</td>
<td>53</td>
</tr>
<tr>
<td>Tie-2 48</td>
<td>ND*</td>
</tr>
<tr>
<td>PDGFR/β 2,820</td>
<td>ND</td>
</tr>
<tr>
<td>EGFR 6,250</td>
<td>ND</td>
</tr>
<tr>
<td>IGF-1R &gt;10,000</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.

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**Fig. 2. Lineweaver Burke plot of ATP competition.** Inhibition of kinase autophosphorylation with CP-547,632. The VEGFR-2 enzyme was incubated with increasing concentrations of ATP and CP-547,632 at room temperature in 96-well plates for 10 min.

**Lineweaver Burke analysis of CP-547632 demonstrating ATP competitiveness.**

**Fig. 3.** Phospho-VEGFR-2 in cell-based assay. CP-547,632 inhibits VEGF-stimulated phosphorylation in cells with an IC\textsubscript{50} value of 6 nM. Porcine aorta endothelial cells stably expressing full-length VEGFR-2 were used in this assay. CP-547,632 was added to serum-deprived cells and incubated at 37°C, 5% for 1 h. The cells were then stimulated with 500 ng/ml VEGF. Whole cell lysates were then immunoprecipitated using anti-VEGFR-2, and Western blot analysis was carried out with antibodies recognizing either the protein or PY (A). Band density was quantitated using a Lumiblaster (B). IP, immunoprecipitate; IB, immunoblot.
sponge in Matrigel. One of each sponge was implanted s.c. bilaterally 1 cm from the central incision on the abdomen of female athymic mice. Three days after surgical implantation, animals received qd oral doses of 100 mg/kg CP-547,632. After 5 days, animals were sacrificed, and sponges were removed. Sponges were ground in 0.1 ml ddH₂O, samples were centrifuged at 15,000 g for 5 min, and hemoglobin content was quantitated by adding 50 μl TMB to 50 μl of the supernatant. The reaction was stopped as described above and absorbance read at 450 nm. Inhibition was calculated as a percentage of the angiogenesis for each growth factor present in the vehicle-treated sponges.

**Tumor Growth Inhibition Studies.** NIH3T3/Hras cells were cultured in DMEM with 10% FBS, and penicillin and streptomycin, in the presence of 0.4 mg/ml G418 (Life Technologies, Inc.). Colo-205 and DLD-1 human colon adenocarcinoma, and MDA-MB-231 breast carcinoma cells were obtained from American Type Culture Collection (Rockville, MD). All of the human cancer cells were propagated by standard tissue culture procedures in the medium suggested by American Type Culture Collection. Exponentially growing cells were trypsinized and resuspended in sterile PBS, and inoculated s.c. (3T3/Hras at 1 × 10⁶ cells/mouse and human tumor cells at 5 × 10⁶ cells/mouse in 200 μl) into the right flank of mice. Animals bearing tumors of approximately 75–150 mm³ in size were divided into groups receiving either vehicle (5% Gelucire) or CP-547,632 (diluted in vehicle) and dosed by oral gavage. Animal body weight and tumor measurements were obtained every 2–5 days. Tumor volume (mm³) was measured with Vernier calipers and calculated using the formula: length (mm) × width (mm) × width (mm) × 0.5. Percentage of growth of an individual tumor was calculated using the following formula: % growth = [(tumor volume (mm³) on final day – tumor volume of day 1) × 100]/tumor volume on day 1. The percentage of growth inhibition was calculated using the following formula: % growth inhibition = ([100 – [(% growth of treated)/% growth of control) × 100]].

**Microscopy and Immunohistochemistry of Tumor Microvasculature.** At the end of the tumor efficacy studies, tumors were isolated and fixed in 4% paraformaldehyde at 4°C for 18–24 h, then processed into paraffin, sectioned at 4 μm, and immunostained for CD31 using an avidin-biotin-peroxidase
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As measured by MTT assay and Western blotting, respectively, indicating that the compound effect was likely mediated by selective kinase inhibition. Furthermore, the CP-547,632 concentrations required to inhibit VEGF-stimulated signaling (IC_{50} = 6 nM) are in agreement with concentrations required to inhibit VEGF-stimulated proliferation of HUVEC (IC_{50} = 14 nM).

In Vivo Inhibition of Receptor Phosphorylation. The in vivo effects of CP-547,632 were characterized by a number of assays to measure modulation of receptor phosphorylation and angiogenic activity, as well as functional effects on tumor growth. In an effort to follow the mechanism of action of this compound in vivo, pharmacokinetic/pharmacodynamic experiments were carried out to measure VEGFR-2 phosphorylation in tumors concomitant with evaluation of drug exposure (plasma concentrations). Because the NIH3T3/H-ras cells used to generate the tumors in mice do not express detectable levels of VEGFR, all of the receptor signal measured is ascribed to the invading murine microvasculature. This model was chosen because it had been reported that H-ras expression leads to an increased expression of VEGF (32) and also because of the consistent nature of tumor

RESULTS AND DISCUSSION

In Vitro Kinase, Cell, and Selectivity Assays. This article describes the pharmacological attributes of a potent and orally bioavailable small molecule inhibitor of the VEGFR TK, CP-547,632 (Fig. 1). This compound is an isothiazole inhibitor of VEGFR-2. Kinetic analyses demonstrate that CP-547,632 is an ATP competitive inhibitor of human VEGFR-2, as increasing amounts of ATP in the reaction were able to restore the kinase V_{max} in the presence of compound (Fig. 2). The enzymatic selectivity of CP-547,632 was assessed by measuring the inhibitory activity of the compound on other purified recombinant human enzymes. Kinase assays were routinely run at ATP concentrations approximating the measured K_{m} for the respective enzymes. CP-547,632 is approximately equipotent (i.e., <10-fold selective) against VEGFR-2, bFGF receptor, and EGFR/Tie-2 chimera, the receptor for Angiopoietin. However, this compound is 250-1000-fold less potent for platelet-derived growth factor receptor β, insulin and the EGF receptor TKs (Table 1).

CP-547,632 inhibits VEGF- and bFGF-stimulated thymidine incorporation in HUVEC with an IC_{50} value of 14 nM and 53 nM, respectively (Table 1). This difference may be because of the stronger proliferative stimulus of bFGF on endothelial cells relative to VEGF. It is unclear whether the modest differences in VEGF, bFGF, and Tie-2 enzyme and cell inhibition are pharmacologically relevant in vivo. We have not pursued in vivo inhibition of Tie-2 with CP-547,632.

To confirm that CP-547,632 inhibits receptor signaling in the cell-based assays, inhibition of VEGF-stimulated VEGFR-2 autophosphorylation was measured in porcine aortic endothelial cells transfected with VEGFR-2. After a 1-h incubation with cells, CP-547,632 inhibited VEGF-stimulated VEGFR-2 phosphorylation in a dose-dependent fashion, with an IC_{50} value of 6 nM (Fig. 3, A and B). Concentrations as high as 1 μM did not affect either cell viability or VEGFR-2 protein amounts as measured by MTT assay and Western blotting, respectively, indicating that the compound effect was likely mediated by selective kinase inhibition. Furthermore, the CP-547,632 concentrations required to inhibit VEGF-stimulated signaling (IC_{50} = 6 nM) are in agreement with concentrations required to inhibit VEGF-stimulated proliferation of HUVEC (IC_{50} = 14 nM).

In Vivo Inhibition of Receptor Phosphorylation. The in vivo effects of CP-547,632 were characterized by a number of assays to measure modulation of receptor phosphorylation and angiogenic activity, as well as functional effects on tumor growth. In an effort to follow the mechanism of action of this compound in vivo, pharmacokinetic/pharmacodynamic experiments were carried out to measure VEGFR-2 phosphorylation in tumors concomitant with evaluation of drug exposure (plasma concentrations). Because the NIH3T3/H-ras cells used to generate the tumors in mice do not express detectable levels of VEGFR, all of the receptor signal measured is ascribed to the invading murine microvasculature. This model was chosen because it had been reported that H-ras expression leads to an increased expression of VEGF (32) and also because of the consistent nature of tumor

![Fig. 6. Effect of CP-547,632 on the growth of human tumors in vivo. A. Colo-205; B. DLD-1; and C. MDA-MB-231 cells were injected s.c into the flank of athymic mice as described in “Materials and Methods.” Animals bearing tumors of 75-150 mm³ each were treated with CP-547,632 (6.25-100 mg/kg, p.o., qd) in 5% Gelucire. The tumors were measured with calipers, and the tumor volumes were calculated as described. Data are plotted as mean; bars, ± SE. qd oral administration of CP-547,632 resulted in the dose-dependent inhibition of tumor growth in all three models. Colo-205 was inhibited 85% with 12 days dosing, whereas the human breast carcinoma MDA-MB-231 achieved a maximum growth inhibition of 80% after 24 days of dosing. Significance was determined using the Student’s t test: * = P < 0.05; ** = P < 0.01, *** = P < 0.001.](image-url)
take rates and growth of this line relative to VEGF-transfected, nontransformed cells (33, 34). The ability to inhibit the phosphorylation of the receptor correlated with blood plasma concentrations of the compound (Fig. 4) demonstrating that this compound inhibits murine, as well as human VEGFR-2 phosphorylation. A single oral dose of 50 mg/kg yielded plasma concentrations above 500 ng/ml for 12 h. This resulted in inhibition of ~50% of measurable VEGFR-2 phosphorylation for 3 h and reductions of at least 30% for up to 12 h. This inhibition corresponded to a C_{average} of 590 ng/ml total drug or 24 ng/ml free drug (~12 nM), correlating well with the cellular IC_{50}. Taken together, these data suggest a correlation among inhibition of VEGFR-2 enzyme activity, in vitro cellular phosphorylation/proliferation, and in vivo tumor vascular VEGFR-2 phosphorylation.

**Inhibition of VEGF and bFGF Induced Angiogenesis.**

CP-547,632 was tested in functional assays measuring corneal angiogenesis, in part to provide an important link between biochemical inhibition (i.e., VEGFR-2 phosphorylation) and functional biological end points (i.e., VEGF-induced angiogenesis). CP-547,632 was shown to inhibit VEGF-induced angiogenesis in vivo in a dose-responsive fashion (Fig. 5). After implantation of the VEGF-hydron pellet, animals received qd doses of CP-547,632 for 5 days. Eyes were examined by slit lamp microscopy, and blood was taken 2 h after the last oral dose. This time point approximates the C_{max} after oral delivery of CP-547,632. A dose of 25 mg/kg, corresponding to a plasma concentration of 106 ng/ml total drug or 42.7 ng/ml free drug (C_{2h}), resulted in 75% inhibition of VEGF-induced angiogenesis relative to vehicle control. CP-547,632 was able to completely inhibit the VEGF-induced corneal angiogenesis at a dose of 100 mg/kg (1884 ng/ml total or 75 ng/ml free drug, C_{2h}). These data together with in vitro and in vivo VEGFR-2 phosphorylation inhibition in xenografts confirm the mechanism-based activity of CP-547,632.

Given the lack of in vitro selectivity between FGFR-2 and VEGFR-2 with CP-547,632, studies were undertaken to evaluate the selectivity in vivo. To this end, a sponge angiogenesis model was

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Fig. 7. The effect of CP-547,632 on the MVD in human colon tumors. After 9–12 days of dosing, tumors were processed for CD31 immunohistochemical detection of endothelium. A significant decrease (25–38%; \( P < 0.05 \)) of MVD in both Colo-205 and DLD-1 tumors was observed after CP-547,632 administration (100 mg/kg, p.o., qd). These tumors were treated identically as those described in the growth inhibition studies, and the MVD was measured at the termination of each experiment. H&E.
used. Animals were bilaterally implanted with a bFGF- and a VEGF-soaked surgical sponge. Animals received qd oral doses of 100 mg/kg CP-547,632 for 5 days resulting in 71% and 89% inhibition of VEGF and bFGF-induced angiogenesis, respectively, when compared with vehicle-treated animals.

**Tumor Growth and MVD Inhibition in Vivo.** The oral antitumor activity of CP-547,632 was additionally examined in several human xenograft models in athymic mice. Mice bearing tumors (75–150 mm$^3$ in size) were randomized into various groups (7–10 animals each) and received CP-547,632 p.o. (6.25–100 mg/kg, qd) for the given number of days. CP-547,632 caused a dose-dependent inhibition of growth in Colo-205, DLD-1, and MDA-MB-231 xenografts (Fig. 6). Oral treatment with 100 mg/kg of CP-547,632 for 9 and 12 days resulted in 69% and 85% inhibition of DLD-1 and Colo-205 tumor growth, respectively. Daily oral administration of CP-547,632 at doses of 100 mg/kg for 24 days was well tolerated (no weight loss, morbidity, or deaths) and produced 80% inhibition of MDA-MB-231 tumor growth. Oral antitumor efficacy of CP-547,632 was also observed in animals treated with 300 mg/kg CP-547,632 for 2 weeks. Proximal tibial growth plate of a control rat (A) and a rat treated with 300 mg/kg CP-547,632 for 2 weeks (B). Note the marked (~6-fold) expansion of the hypertrophic zone of the epiphyseal cartilage in the treated rat at this dose. H&E, ×40.
bearing EBC-1 and H460, human non-small cell lung carcinoma tumors. Approximately 50% tumor growth inhibition was achieved with 100 mg/kg, qd, oral dosing with CP-547,632 (data not shown). In the models described here, administration of CP-547,632 as a single agent did not yield tumor regressions. However, when CP-547,632 was administered in combination with conventional cytotoxic drugs, tumor regressions were observed (data not shown).

Although data demonstrate that CP-547,632 inhibits VEGF-induced angiogenesis in vivo (Fig. 5), additional investigations were undertaken to examine whether this compound affected microvascular density in the human tumor models. To this end, DLD-1 and Colo-205 tumors from growth inhibition studies were processed for immunohistochemical detection of endothelium using CD31 Abs (Fig. 7). The data demonstrate a statistically significant decrease of 38% in the number of microvessels in vehicle-treated versus drug-treated DLD-1 tumors (100 mg/kg was the dose that resulted in 69% tumor growth inhibition reported above). It should be noted that the modifications to tumor vascular morphology and function in response to VEGFR inhibitors can be complex. Using PTK787, it was found that although vascular density was reduced in tumors, there was an intriguing increase in partial blood volume; and whereas vascular permeability was decreased significantly, there were no measurable changes in extravasation (35).

Inhibition of Endochondral Bone Growth. VEGF has also been implicated in promoting the microvascular invasion required for normal endochondral bone growth (36). An additional indication of an antiangiogenic effect of CP-547,632 was demonstrated in immature rat tibial growth plates (Fig. 8). Treatment of rats with CP-547,632 for 30 days at doses ≥10 mg/kg produced widening of the growth plate. The hypertrophic zone of the epiphyseal cartilage was affected predominantly, exhibiting up to ~10-fold increases in thickness. Similar observations have also been made with ZD-6474, a small molecule VEGFR inhibitor (37), as well as with bevacinumab (38).

A number of VEGFR signaling inhibitors are being developed as angiogenesis inhibitors (39). On the basis of the preclinical data implicating an important role for VEGFR in angiogenesis, VEGFR inhibitors represent one of the most promising classes of antiangiogenesis agents (reviewed in Ref. 40). There are a variety of compounds in clinical development, both small molecules (37, 41) and biologicals (23, 42, 43). Key distinctions among the small molecules described to date include the pharmacology of the compounds as well as the enzyme selectivity profiles. For example, ZD-6474 from Astra Zeneca appears to have activity for VEGFR-2 and EGF receptor, whereas PTK787 is a sub-1 μM inhibitor of VEGFR-2, PDGFR, and c-kit. CP-547,632 is a potent inhibitor of VEGFR, TK with immunoglobulin and epidermal growth factor homology domains 2, and FGFR-2 in various kinase, cell, or in vivo assays. Because of this selectivity pattern, the overall tumor growth inhibition observed with CP-547,632 may be a combination of any of these three targets. Moreover, deducing the role that any of these receptors have in inhibiting tumor cell or vessel growth is additionally complicated because different tumor models may have different susceptibilities to each target. These small molecule angiogenesis inhibitors can be tools to help dissect and understand tumor vascular biology; however, no compound to date has demonstrated specificity for a single growth factor receptor. Although some inhibitors demonstrate modest kinase selectivity in vitro, studies to confirm pharmacological selectivity in vivo require rigorous assessment of plasma and tumor concentrations of the compound or side-by-side comparison of target inhibition in the same animal model. These data are critical for the accurate interpretation of results and associating inhibition of a particular receptor with the resulting biological response. Failure to adequately characterize the in vivo pharmacology will invariably lead to erroneous conclusions about the role of particular targets and vascular cell types involved in the angiogenic process.

In this article, we describe the identification and characterization of a novel TK inhibitor, CP-547,632. This compound is an ATP-competitive and reversible inhibitor of the VEGFR-2 kinase that potently inhibits receptor phosphorylation in vitro and in vivo. Plasma concentrations of this compound that result in 50% inhibition of receptor tyrosine phosphorylation in tumor xenografts achieve 50% inhibition of VEGF-induced angiogenesis in the murine corneal implant model. Additionally, this compound potently inhibits both VEGF- and bFGF-induced angiogenesis. Finally, qd oral administration of this compound was able to significantly inhibit human tumor growth in murine models resulting in significant inhibition of tumor microvascular density in vivo. Taken together, these results demonstrate CP-547,632 to be a potent angiogenesis inhibitor with good oral bioavailability and in vivo antitumor efficacy. CP-547,632 represents an exciting new chemotype for clinical development in the area of VEGFR inhibitors.

Clinical testing with this and other inhibitors will be required to relate potency, duration of pharmacokinetic/pharmacodynamic effect, and the spectrum of enzymatic selectivity to the safety and efficacy profile that provides optimal benefit for cancer patients.

ACKNOWLEDGMENTS

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REFERENCES


Pharmacological Characterization of CP-547,632, a Novel Vascular Endothelial Growth Factor Receptor-2 Tyrosine Kinase Inhibitor for Cancer Therapy

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