CEP-7055: A Novel, Orally Active Pan Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases with Potent Antiangiogenic Activity and Antitumor Efficacy in Preclinical Models

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ABSTRACT

Inhibition of the vascular endothelial growth factor VEGF-VEGF receptor (VEGF-R) kinase axes in the tumor angiogenic cascade is a promising therapeutic strategy in oncology. CEP-7055 is the fully synthetic orally active N,N-dimethylene glycerol ester of CEP-5214, a C3-isopropylmethylene) fused pyrrolocarbazole with potent pan-VEGF-R kinase inhibitory activity. CEP-5214 demonstrates IC50 values of 18 nm, 12 nm, and 17 nm against human VEGF-R2/KDR kinase, VEGF-R1/FLT-1 kinase, and VEGF-R3/FLT-4 kinase, respectively, in biochemical kinase assays. CEP-5214 inhibited VEGF-stimulated VEGF-R2/FLT4 autophosphorylation in human umbilical vein endothelial cells (HUVECs) with an IC50 of ~10 nm and demonstrated an equivalent inhibition of murine FLK-1 autophosphorylation in transformed SVR endothelial cells. Evaluation of the antiangiogenic activity of CEP-5214 revealed a dose-related inhibition of microvessel growth ex vivo in rat aortic ring explant cultures and in vitro on HUVEC capillary-tube formation on Matrigel at low nanomolar concentrations. The antiangiogenic activity of CEP-5214 in these bioassays was observed in the absence of apparent cytotoxicity. Single-dose p.o. or s.c. administration of CEP-7055 or CEP-5214 to CD-1 mice at 23.8 mg/kg/dose b.i.d. resulted in a reversible inhibition of VEGF-R2/FLK-1 phosphorylation in murine lung tissues. Administration p.o. of CEP-7055 at 2.57 to 23.8 mg/kg/dose b.i.d. resulted in dose-related reductions in neovascularization in vivo in porcine aortic endothelial cell (PAEC)-VEGF/ basic fibroblast growth factor-Matrigel implants in nude mice (maximum, 82% inhibition), significant reductions in granuloma formation (30%) and granuloma granularity (42%) in a murine chronic inflammation-induced angiogenesis model, and significant and sustained (6 h) inhibition of VEGF-induced plasma extravasation in rats, with an ED50 of 20 mg/kg/dose. Chronic p.o. administration of CEP-7055 at doses of 11.9 to 23.8 mg/kg/dose b.i.d. resulted in significant inhibition (50–90% maximum inhibition relative to controls) in the growth of a variety of solid and hematopoietic tumor types (1–4). The antitumor efficacy of chronic CEP-7055 administration was independent of initial tumor volume (in the ASPC-1 pancreatic carcinoma model) and reversible on withdrawal of treatment. Chronic p.o. administration of CEP-7055 in preclinical efficacy studies for periods of up to 65 days was well tolerated with no apparent toxicity or significant morbidity. Orally administered CEP-7055 has entered Phase I clinical trials in cancer patients.

INTRODUCTION

Angiogenesis, the development of new blood vessels from the endothelium of a preexisting vasculature, is a critical process required by most solid tumors to support their localized growth and metastatic dissemination within the host (1–4). The autocrine, paracrine, and amphiregulin interactions of the vascular endothelium with its surrounding stromal components, as well as with the proangiogenic and angiostatic cytokines and growth factors orchestrating physiological angiogenesis, are tightly regulated both spatially and temporally. In contrast, the pathological angiogenesis necessary for active tumor growth is sustained and persistent, with the initial acquisition of the angiogenic phenotype being a common mechanism for the development of a variety of solid and hematopoietic tumor types (1–4).

Among the known angiogenic growth factors and cytokines implicated in the modulation of normal and pathological angiogenesis, the VEGF3 family (VEGF-A, VEGF-B, VEGF-C, VEGF-D) and their corresponding receptor tyrosine kinases [VEGF-R1 (FLT-1), VEGF-R2 (FLK-1, KDR), and VEGF-R3 (FLT-4)] play a paramount and indispensable role in regulating the multiple facets of the angiogenic and lymphangiogenic processes [VEGF-R3 (FLT-4)], as well as the induction of vascular permeability and inflammation (1, 2, 5–7). In contrast to pleiotropic angiogenic factors and cytokines, the VEGF family has a relatively narrow target cell specificity and exert their mitogenic, chemotactic, and thrombogenic effects, primarily on endothelial cells implicated in hemangiogenesis (VEGF-A, VEGF-B) and lymphangiogenesis (VEGF-C and VEGF-D; 2, 5–7). The endothelial-specific activities of the VEGF family are mediated through the receptor tyrosine kinases: VEGF-R1/FLT-1, VEGF-R2/KDR, and VEGF-R3/FLT-4, the expression of which is up-regulated on vascular endothelial cells during embryonic and tumor angiogenesis (2–5). The receptor VEGF-R2/KDR is the principal one through which VEGFs exerts their mitogenic, chemotactic, and vascular permeabilizing ef

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1 The pharmacological development of CEP-7055 is being pursued in the context of a partnership agreement between Cephalon, Inc. (West Chester, PA) and Sanofi-Synthelabo (Gentilly, France).

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGF-R, VEGF receptor; FGF, fibroblast growth factor; FGF-R, FGF receptor; bFGF, basic FGF; HUVEC, human umbilical vein endothelial cell; PDGF-R, platelet-derived growth factor receptor; PLC-γ, phospholipase C-γ; GST, glutathione S-transferase; RENCA, (murine) renal cell carcinoma (model); IMD, intratumoral microvessel density; MED, minimum effective dose; TCA, trichloroacetic acid; PAEC, porcine aortic endothelial cell; IL, interleukin.
fects on the host vasculature (7–10). Increased expression of VEGFs by tumor cells and VEGF-R2/KDR and VEGF-R1/FLT-1 by the tumor-associated vasculature are a hallmark of a variety of human and rodent tumors in vivo and correlated with tumor growth rate, microvessel density/proliferation, tumor metastatic potential, and poorer patient prognosis in a variety of malignancies (1–6).

Evidence exists for the importance of VEGF-R1 and its ligands, VEGF and placental growth factor (PIGF), in modulating KDR/VEGF-R2 activity (9–12), mediating chemotactic activity in monocytes and macrophages (13, 14), regulating extracellular matrix proteolytic activity (15) and the release of tissue factor and nitric oxide in endothelial cells and trophoblasts, respectively (12, 13), and in mediating placental growth factor-induced recruitment and mobilization of bone-marrow derived endothelial and hematopoietic stem cells in normal and pathological (tumor-associated) angiogenesis (16, 17).

Similarly, a growing body of evidence implicates VEGF-R3/FLT-4 and its ligands, VEGF-C and VEGF-D, in the induction of tumor lymphangiogenesis and lymphatic metastases in multiple solid tumor types (18–22). These collective findings and the critical and non-redundant role of VEGF in tumor-associated angiogenesis (23, 24) support the importance of the selective abrogation VEGF-mediated signaling events through all three VEGF-R subtypes as an optimal therapeutic strategy in the management and treatment of a variety of solid and hematopoietic tumors.

Angiogenesis therapies directed against the VEGF-VEGFR kinase axes through a variety of approaches have been a promising and well-validated therapeutic approach under active evaluation for their safety and efficacy in multiple clinical trials (25–27). The preclinical biochemical, pharmacological and in vivo efficacy profile of antibody-, soluble receptor-, and ribozyme-based angiogenic therapies currently under clinical evaluation have been described extensively (see 25–33). In addition, several orally active small molecule inhibitors of specific VEGF-R kinases (see Ref. 34), including PTK-787 (vatalanib; Refs. 35–38), PKC-412 (midostaurin; Ref. 39), ZD 6474 (40, 41), SU-11248 (42, 43), and CP-547,632 (44) are currently under clinical evaluation.

In this report, we describe the biochemical and pharmacological profile of CEP-7055, the N,N-dimethyl glycine ester prodrug of CEP-5214, a novel, orally active and fully synthetic low nanomolar inhibitor of all three VEGF-R kinases (Table 1). Its ester derivative, CEP-7055, was prepared to increase aqueous solubility and to facilitate p.o. delivery. CEP-7055 demonstrates a broad acting preclinical antitumor and angiogenic efficacy and a tolerability profile amenable for chronic p.o. administration. CEP-7055 is currently in Phase I trials in patients with solid tumors.

MATERIALS AND METHODS

Compound Synthesis

CEP7055, N,N-dimethylglycine 3-[[5,6,7,13-tetrahydro-9-[1-(methylthoxy)methyl]-5-oxo-12H-indeno[2,1-a]pyrrolo[3,4-c]carbazol-12-yl]propyl ester, the ester prodrug of CEP-5214, was synthesized in the Department of Chemistry at Cephalon, Inc. (West Chester, PA). The synthetic routes for CEP-7055 and CEP-5214 have been described elsewhere (45, 46). For all in vivo experiments described with CEP-7055, the HCl salt was used at ≈97% purity. A dose of 1.19 mg/kg CEP-7055 prodrug is equivalent to 1.0 mg/kg dose of CEP-5214.

Cell Lines

The majority of rodent and human tumor cell lines used in these studies were obtained from the American Type Culture Collection (Manassas, VA). The Dunning G/VEGF165 rat prostate carcinoma and LNCaP human prostate carcinoma cell lines were obtained from Dr. Roberto Pili (Johns Hopkins University, Baltimore, MD), and cultures of human U251MG and SF 776 glioblastoma cells were obtained from the Brain Tumor Research Center Tissue Bank (University of California, San Francisco, CA). All of the cell lines were MAP-16- and Mycoplasma-tested by a commercial laboratory (Bio Reliance Corp., Rockville, MD) and were deemed suitable for in vivo studies. HUVECs were obtained from Clonetics (San Diego, CA) and cultured in endothelial cell basal medium (EBM-2; Clonetics) with 2% heat-inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY), 50 μg/ml endothelial cell growth supplement, 50 μg/ml heparin, 10 mM HEPES, and 2 mM t-glutamine. Cells between passages 3 and 8 were used as described below (“Human and Murine Endothelial Cell-based Receptor Phosphorylation Assays” and “In Vitro Capillary Tube Formation Assay with HUVECs on Matrigel”).

Animals

Female and athymic nu/nu mice (6–8 weeks old; Charles River, Wilmington, MA) were maintained five/cage in microisolator units on a standard sterilizable laboratory diet (Teklad Labchow; Harlan Teklad, Madison, WI). Animals were housed under humidity- and temperature-controlled conditions, and the light/dark cycle was set at 12-h intervals. Out-bred Balb/C mice of 6–8 weeks of age were obtained from Charles River (Wilmington, MA) and female out-bred Tuck Original mice were obtained from Harlan (Oxon, United Kingdom). The animals were housed in groups of 12 mice per cage in a room with a 12-h light/dark cycle. The mice were fed ad libitum normal mouse chow pellets. Male Sprague Dawley rats (250–300 g) were obtained from Charles River and housed five/cage in a conventional vivarium facility. All mice and rats were quarantined 1 week before experimental manipulation. All of the animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committees (IACUC) of Cephalon and Sanofi-Synthelabo.

Recombinant Proteins and Biochemical Kinase Assays

Enzymes and Substrates. The cytoplasmic domains of recombinant human tyrosine kinases (epidermal growth factor receptor, FGFR1, FLT-3, PDGF-Rβ, TIE-2, TRKA, VEGF-R1, VEGF-R2, VEGF-R3) and serine/threonine kinases (CHK1, CDK1/cyclinB, CDS1, DLK, JNK1/2/3, MLK1, MLK2, MLK3) were expressed in a baculovirus insect cell system. Recombinant β-insulin receptor kinase was purchased from Stratagene (La Jolla, CA). Purified rat brain protein kinase C (mixture of Ca2+-dependent isoforms α, β, and γ) and activated recombinant human GST-p38α were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). PLC-γ was generated as a fusion protein with GST following the procedure of Rotin et al. (47). Histone H-1 and MBP were purchased from Fluka Chemical Corp. (Milwaukee, WI) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. Time-resolved fluorescence experiments were performed using Eu-N1 antiphosphotyrosine antibody, Eu-N1 antiphosphothreonine antibody, Eu-N1 antirabbit IgG, and DELFIA enhancement solution purchased from Perkin-Elmer Life Sciences (Gaithersburg, MD). Phospho-specific antibodies to ATF-2 (Thr71), Cdc25c (Ser216), and Rb (Ser795) were obtained from New England Biolabs, Inc. (Beverly, MA).

Receptor-linked Tyrosine Kinase Assays. Enzyme-inhibition studies were performed using a modification of the ELISA described for TRKA kinase (48). Briefly, the 96-well microtiter plate (FluoroNUNC or Costar High Binding) was coated with 10 μg/ml recombinant human PLC-γ/GST. Kinase assays were performed in 100 μl reaction mixtures containing 50 mM HEPES (pH 7.4), Km level of ATP, 10 mM MgCl2, 0.1% BSA, 2% DMSO, and various concentrations of CEP-5214 or CEP-7055. The reaction was initiated by adding baculoviral recombinant human enzyme (epidermal growth factor receptor, FGFR1, β-RK, PDGF-Rβ, FLT-3, TIE-2, TRKA, VEGF-R2, or VEGF-R3) and was allowed to proceed at 37°C for 15 min at 37°C. The detection antibody, Eu-N1 antiphosphotyrosine (PT66) antibody was added. After a 1-h incubation at 37°C, 100 μl of enhancement solution was added and the plate was gently agitated. After 5 min, the fluorescence of the resulting solution was measured using the Victor2 Multilabel Counter (Model 1420-018). The activities of the serine/threonine kinases listed in Table 1 were measured using either a radioactive Multiscreen TCA “in-plate” assay (49) or an ELISA similar to that described above for the tyrosine kinases.

Data Analysis. Inhibition curves for compounds were generated by plotting percentage control activity versus log10 of the concentration of compound. IC50 values were calculated by nonlinear regression using the sigmoidal
dose-response (variable slope) equation in GraphPad Prism. IC_{50} values were reported as the average of at least three separate determinations.

**Human and Murine Endothelial Cell-based Receptor Phosphorylation Assays**

Subconfluent HUVECs were serum starved by replacing medium with EBM-2 (endothelial cell basal medium, serum-free; Clonetics) containing 0.05% BSA for 1 h at 37°C, during which time, a range of concentrations of CEP-5214 or DMSO (control) was added to the cells. Human VEGF (Clonetics) was then added to HUVECs at a concentration of 10 ng/ml for 5 min. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 1 mM activated sodium vanadate and protease inhibitors (Protease Inhibitor Cocktail Set III; Calbiochem, San Diego, CA), sheared with a 27-gauge syringe, and then centrifuged at 12,000 × g for 15 min. Clarified cell lysates were immunoprecipitated with anti-VEGF-R2 antibody (CEP-133) for 1 h, followed by incubation with Protein A-Sepharose for another hour at 4°C and analysis by SDS-PAGE as detailed previously (49, 50). Phosphorylated proteins were visualized using enhanced chemiluminescence (Amersham, Piscataway, NJ). The percentage inhibition was calculated by analyzing scanned autoradiographs on a densitometer. Scores were based on decrease in protein band density compared with VEGF-stimulated control (no inhibitor) as follows: 0, no decrease; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%. Murine SVR-transformed endothelial cells (51) were treated in the same way as HUVECs with the following modifications: subconfluent cells were serum starved for 1 h at 37°C with DMEM containing 0.05% BSA; in addition, autophosphorylation of murine FLK-1 was measured after stimulation for 5 min with recombinant murine VEGF (R&D Systems, Minneapolis, MN).

**In Vitro Capillary Tube Formation Assay with HUVECs on Matrigel**

The ability of CEP-5214 to inhibit angiogenesis in vitro was evaluated in a capillary tube formation assay using HUVECs cultured on a synthetic basement membrane matrix (52–55). Under these conditions, HUVECs are capable of morphological differentiation into an extensive network of capillary-like structures composed of highly organized three-dimensional cords (55). Forty-eight-well Nunclon (Fisher Scientific, Newark, DE) plates were coated with a 200-μl (2 mg) layer of the synthetic basement membrane substrate Matrigel (Collaborative Research, Bedford, MA) at 10 mg/ml concentration and were incubated at 37°C for 30 min to promote gelling. HUVECs (Clonetics) were cultured in EMB-2 medium (Clonetics) with 2% fetal bovine serum, and cells between passages 3 and 8 were seeded (at 3 × 10^4 cells in 200 μl of medium) in each of the Matrigel-coated wells. CEP-5214 was diluted in DMSO to a final DMSO concentration in the treated and untreated wells of 0.02%. After an 18-h incubation at 37°C and 5% CO₂ humidified atmosphere, HUVECs were aspirated from the medium and were fixed and stained using a modified Wright-Giemsa staining protocol according to the manufacturer’s recommendations (Diff-Quik Stain Set; Baxter Healthcare Corp., McGraw Park, IL). Complete capillary tube networks within a designated area of a low magnification (×10) field were counted under light microscopy, and the data were expressed as percentage of complete capillary tube formation relative to untreated HUVEC control cultures incubated under the same conditions (54, 55). All of the assays were done in quadruplicate in three independent experiments. Statistical analysis of the inhibition of tube formation relative to control cultures was done by the Dunnet’s multiple-comparison test, with P < 0.05 designated as significant. Visual inspection of cultures on a routine basis was used to assess the potential cytotoxicity of CEP-5214 relative to control cultures.

**Ex Vivo Rat Aortic Ring Explant Assay in Collagen Gel Matrices**

Rat aortic ring explant cultures were prepared by a modification of protocols previously described (54, 56–58). This assay enables a quantitative assessment of microvessel growth, branching, and remodeling; and vessel regression in a primary explant culture system in which endothelial cell-adenitial cell interactions critical for angiogenesis in vivo can be effectively modeled in a more physiologically relevant ex vivo setting. CEP-5214 was dissolved in DMSO and mixed with serum-free EBM immediately before the addition or replacement of media to collagen-embedded aortic ring explant cultures in quadruplicate. The final DMSO concentration in treated and control cultures was 0.02%. Cultures were incubated at 35.5°C in a humidified CO₂ atmosphere, and the medium was replaced daily over the course of the 8- to 10-day studies. Visual counts of microvessel outgrowths from replicate explant cultures (n = 8) were done under bright-field microscopy following an established protocol (57, 58). Experiments were done three times, and microvessel counts in treated and control cultures were analyzed by one-way ANOVA and the Student-Newman-Keuls multiple-comparison test, with P < 0.05 deemed significant.

**Pharmacokinetics of P.o. CEP-7055 Administration in Mice**

Single-dose p.o. administration of CEP-7055 was performed in female CD-1 mice and female athymic nude mice of 6–8 weeks of age. A dose of 1.19 mg/kg CEP-7055 is equivalent to 1.0 mg/kg dose of CEP-5214. Trunk blood from mice on day 7 following the given the HCl salt of CEP-7055 p.o. was collected after decapitation using heparin as the anticoagulant. Plasma was prepared for high-performance liquid chromatography (HPLC)/mass spectrometric analysis by protein precipitation with two volumes of acetonitrile (200 μl) per 100-μl sample of plasma. Pharmacokinetic parameters were determined by WinNonLin software.

**Pharmacodynamic Effects of CEP-7055 on VEGF-R2/FLK-1 Phosphorylation in Vivo in Mice**

Pharmacodynamic effects of CEP-7055, or direct administration of CEP-5214, were evaluated as described by others (43, 44). Briefly, athymic nude mice bearing established VEGF-R2 expressing (52) SVR angiosarcomas (~250 mm³) were administered a single p.o. or s.c. dose of CEP-5214 at a 23.8 mg/kg/dose, and tumors were excised 30–45 min later. Tumor lysates were prepared and the tyrosine autophosphorylation of the VEGF-R2/FLK-1 receptor was analyzed by immunoprecipitation with SC-504 and SC-315G anti-FLK-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and by Western blotting with 4G10 antiphosphotyrosine antibody as described above, which confirmed the detection of a ~220,000 protein of the expected size. Immunobioabsorption with increasing concentrations of blocking peptides to these antisera confirmed the specificity of the immunoprecipitation for murine FLK-1 receptor in SVE cells and tumors. In related studies, athymic mice received a single p.o. dose of CEP-5214 at 39 mg/kg/dose or CEP-5214 directly at a 50-mg/kg/dose. At 20, 40, and 60 min after dosing, animals were given murine VEGF at 3 ng/mouse i.v. and were sacrificed 5 min later; protein extracts were then prepared from lungs. Tyrosine phosphorylation of FLK-1 was evaluated by Western blotting as above, using PC460, a phospho FLK-1 antibody. The abundance of VEGF-R2/FLK-1 in each sample lysate was assessed by Western blotting, and the samples were evaluated densitometrically.

**PAEC-VEGF/bFGF-induced Matrigel Implant in Vivo Angiogenesis Model in Nude Mice**

The Matrigel plug implantation assay used in these studies was a modification of that described previously (59, 60). Briefly, PAECs were grown to confluency in Ham’s F-12 medium supplemented with 10% fetal bovine serum. Cells were used between passages 5 and 10. Nude mice were given bilateral s.c. injections of 0.5 ml Matrigel synthetic basement membrane (Collaborative Research) containing 1 × 10^6 PAECs/plug and recombinant murine VEGF and bFGF (R&D Systems) at 20 ng/ml and 250 ng/ml, respectively, final concentrations per plug. Mice bearing PAEC-VEGF/bFGF-Matrigel implants were randomized into groups (10/group) and were given doses of CEP-7055 or vehicle (1% aqueous acetic acid) p.o. b.i.d. for 8 days. The hemoglobin content of the PAEC-VEGF/bFGF-Matrigel plugs has been reported to be directly proportional to the degree of neovascularization in each plug (59) and was determined as described previously (60). Contralateral plugs were evaluated histologically for vessel morphology. Results from duplicate in vivo experiments are expressed as mean g/dl of hemoglobin ± SE. Statistical analyses of the data were done using the paired Student’s t test, with P < 0.05 deemed significant.

**Chronic Inflammation-induced in Vivo Angiogenesis Model in Mice**

Angiogenesis was induced in female out-bred Tuck Original mice (25 g initial body weight) with a chronic granulomatous reaction to Freund’s complete adjuvant in croton oil as described previously (61). Briefly, granuloma-
tous air pouches were induced by the s.c. injection of 3 ml of air into each anesthetized mouse and by the injection of 0.5 ml of Freund’s complete adjuvant with 0.1% croton oil 4–5 h later. Doses of CEP-7055 were administered p.o. b.i.d. in 1% aqueous acetic acid over a 6-day period, and the degree of inflammation and vascular density were evaluated on day 7. The vascular content was assessed by the formation of vascular casts incorporating carmine. Mice were anesthetized with pentobarbital (60 mg/kg/dose, i.p.) and peripheral vasodilatation induced by placing mice on a heated pad at 40°C for 10 min. The cast was formed by the i.v. injection of one ml of 5% carmine red in 10% gelatin into the warmed mice. The carcasses were chilled, the granulomatous air pouch linings were dissected, and the tissues were dried, enzymatically digested, and analyzed spectrophotometrically as described previously (61).

**VEGF-induced Plasma Extravasation Model in Rat Skin**

Male Sprague Dawley rats (270 to 320 g) were anesthetized with sodium pentobarbital at 60 mg/kg/dose i.p. Animals were given i.v. injections of Evans blue dye (15 mg/kg/dose), and, 5 min later, saline solution (NaCl 0.9%) or VEGF (3 ng) were injected intradermally (0.1 ml/site). Four paired injections of saline solution and VEGF were performed on the back of each rat. Measurements of the vertical and the horizontal diameter of the blue area were performed for each injection point, and the area of plasma extravasation was calculated (radius vertical × radius horizontal × π). Basal plasma extravasation (0.9% saline solution) was not modified by the treatment with CEP-7055 at any time or at any dose tested. Consequently, for each VEGF/saline solution pair, the basal extravasation area corresponding to the injection of saline solution was subtracted from the extravasation area that resulted from the VEGF injection. Administration p.o. of CEP-7055 or vehicle (1% aqueous acetic acid) was done over a 6-h time course. For dose-response studies, CEP-7055 was administered 60 min before the measurement of local plasma extravasation at the indicated doses (see Fig. 7). The corrected areas of the CEP-7055 treated group were compared with the vehicle group with Student’s unpaired t test, with P < 0.05 deemed significant.

**Human and Murine s.c. Tumor Xenograft Models in Nude Mice**

The p.o. antitumor efficacy of CEP-7055 was evaluated in multiple murine and human tumor xenograft models on therapeutic dosing regimens, i.e., administration of CEP-7055 to athymic nude mice bearing established palpable s.c. tumor xenografts. Female athymic nu/nu mice (6 to 8 weeks old) were maintained five/cage in microisolation units on a standard sterilizable laboratory diet (Teklad Labchow). Mice were quarantined 1 week before experimental maintenance five/cage in microisolator units on a standard sterilizable laboratory diet (Teklad Labchow). Mice were quarantined 1 week before experimental administration of CEP-7055 to athymic nude mice bearing established palpable xenografts. Female athymic nu/nu mice (6 to 8 weeks old) were maintained five/cage in microisolation units on a standard sterilizable laboratory diet (Teklad Labchow). Mice were quarantined 1 week before experimental administration of CEP-7055 to athymic nude mice bearing established palpable xenografts. Female athymic nu/nu mice (6 to 8 weeks old) were maintained five/cage in microisolation units on a standard sterilizable laboratory diet (Teklad Labchow). Mice were quarantined 1 week before experimental administration of CEP-7055 to athymic nude mice bearing established palpable xenografts.

**Orthotopic Prostate and Renal Carcinoma Tumor Models in Mice**

The antitumor efficacy of CEP-7055 was further evaluated in orthotopic models of hormone-dependent human prostate carcinoma in male nude mice (60) and in the Renca in BALB/c mice (35, 36, 63) to assess its efficacy on primary, local invasive, and metastatic tumor growth in both immunocompromised and immunocompetent mice, respectively. Briefly, experimentally growing human androgen-sensitive LNCaP prostate carcinoma cells were harvested from tissue culture plates by trituration. LNCaP cells were injected orthotopically (2 × 10^5 cells/20 μl per mouse) into the prostate of athymic nude male mice (6–8 weeks old). Treatment with CEP-7055 was initiated 1 day after orthotopic implantation of LNCaP cells and continued for 21 days, at which time animals were euthanized by CO₂ asphyxiation and tumors were analyzed (mean wet tumor weight in mg ± S.E.). Administration p.o. of CEP-7055 was evaluated in an orthotopic murine renal carcinoma (RENCA) metastasis model in syngeneic BALB/c mice, essentially as described for the evaluation of other antiangiogenic agents (35, 36, 63). For antitumor efficacy studies, RENCA cells (1 × 10^5 in 50 μl of sterile 1 × PBS) were injected into the renal subcapsular space of the left kidney of female BALB/c mice. Two days after recovery from surgical implantation, groups of mice were randomized into treatment groups and were given CEP-7055 over a 21- to 26-day period. Human recombinant IL-2 (Proleukin; Chiron Corp, Emeryville, CA) was used as a reference standard in the Renca model using previously established dosing protocols of 30,000 units/dose i.p. for 5 days/week (64). Mice were necropsied for gross assessment of local and disseminated tumor burden (lungs, regional lymph nodes) and histopathological analysis of selected tissues. A metastatic scoring system was used to evaluate the local and metastatic tumor burden in these studies: I, primary mass with 0–10 nodules in the lung; II, primary mass; 10–100 nodules in the lung; III, primary mass and too numerous to count (TNTC) nodules in the lung but still normal areas of lung visible; IV, primary mass with TNTC nodules in the lung with no normal lung visible. Statistical analyses of tumor and lung weights were done by the Mann-Whitney rank-sum test, with P < 0.05 deemed significant.

**Tumor Histological and Immunohistochemical Analyses of MicrovesSEL Density**

In situ evaluation of IMD in tumor xenografts was determined immunohistochemically by evaluating expression of the endothelium-associated antigen, von Willebrand factor (Factor VIII) and CD34 immunostaining for confirmation) by modification of methods described previously (24, 65). Tissue sections were processed, deparaffinized, rehydrated, and quenched for endogenous peroxidase activity as described previously (65). After a 10-min permeabilization step with protease K, murine vessels in tumor xenografts were immunostained with rabbit anti-von Willebrand factor (DAKO Corp., Carpentrya, CA) at 20 μg/ml or anti-CD34 (DAKO Corp.) at 1:2000 for 18 h at 4°C. Sections were incubated 30 min at room temperature with biotin-labeled goat antirabbit IgG after incubation with streptavidin-horseradish peroxidase (VectorStain Elite; Vector Laboratories, Burlingame, CA). Sections were developed after washing with diaminobenzidine (DAB) as a chromogen and counterstained with 1% methyl green. For quantification of IMD, 10 fields (97,500 μm²/field) of five tumors were evaluated in a blinded fashion at ×100 magnification, and the percentage inhibition of IMD relative to vehicle-treated control tumors was determined. Statistical analyses of IMD in tumor xenografts from CEP-7055-treated mice compared with vehicle-treated mice were done using a two-tailed Student’s t test, with P < 0.05 deemed significant.

**Calcein-AM Cell Proliferation Assays**

Calcein-AM cell proliferation assays were conducted as described previously (62) in a 96-well format to assess direct effects of CEP-5214 at 24- and 48-h incubation on the proliferative fraction of select tumor cell lines. Plates were read on a CytoFluor 2300 fluorescence plate reader at various sensitivities with an excitation wavelength of 485 nm and emission wavelength of 530 nm. Negative control wells contained medium, but no cells, and were assayed with calcein, as described above. All of the studies were conducted twice and in triplicate for each sample concentration.
RESULTS

Kinase Inhibition Profile of CEP-7055 and CEP-5214

The effects of CEP-5214 and its pro-drug CEP-7055 (Fig. 1) were evaluated in enzyme-based assays for the inhibition of VEGF-R2 kinase activity. The primary assay for identifying inhibitors of the VEGF-R2 kinase activity uses an ELISA-based assay with time-resolved fluorescence readout and uses recombinant human PLC-γ1/GST fusion protein as a substrate. To interpret the inhibition data for CEP-7055, although the ester is slightly less potent than the parent compound (VEGF-R1/FLT-1 IC₅₀ 16 nM; VEGF-R2/KDR IC₅₀ 8 nM; VEGF-R3/FLT-4 IC₅₀ 4 nM). The same rank order of inhibition was observed for CEP-7055, although the ester is slightly less potent than the parent compound (VEGF-R1/FLT-1 IC₅₀ 57 nM; VEGF-R2/KDR IC₅₀ 18 nM; VEGF-R3/FLT-4 IC₅₀ 8 nM). The Hill slopes for CEP-5214 and CEP-7055 inhibition of human VEGF-R2/KDR kinase are shown in Fig. 2. CEP-5214 demonstrates a somewhat weaker inhibition of the human c-Kit, PDGF-RB, and FGFR1 kinases, with IC₅₀ values of 57, 128, and 162 nM, respectively, and is a potent inhibitor of MLK 1, 2, 3, and 6, with IC₅₀ values of 13, 46, 7, and 240 nM, respectively. CEP-5214 is largely inactive against the remainder of the tyrosine and serine/threonine kinases evaluated in this enzyme-based assay (Table 1).

Effects of CEP-5214 on VEGF-induced VEGF-R Phosphorylation in Human and Murine Endothelial Cells

The VEGF-R kinase inhibitory activity of CEP-5214 was evaluated for its dose-related inhibition of VEGF-R2/KDR autophosphorylation in cell-based assays using HUVECs and murine SVR endothelial cells and monitoring the inhibition of VEGF-induced stimulation of VEGF-R2/KDR phosphorylation. The estimated IC₅₀ of CEP 5214 in these cellular assays was ~10 nM with both HUVEC and SVR cells and compared closely with its observed IC₅₀ in enzyme-based assays (4–14 nM; Table 1), indicating that this compound is highly cell permeable and essentially equivalent in its inhibition of human and murine VEGF-R2 receptor kinase activity. At concentrations of CEP-5214 of 100 nM or greater, there was complete inhibition of VEGF-stimulated VEGF-R2 phosphorylation within a 15-min exposure of both human and murine endothelial cells in vitro. In a wash-out assay conducted to determine the duration of the inhibitory effects of CEP-5214 on VEGF-stimulated VEGF-R2 phosphorylation, inhibition of VEGF-R2/KDR phosphorylation in vitro was sustained maximally for 1 h after CEP-5214 exposure, was still apparent 3 h after exposure, and declined to basal levels by 6 h postexposure; results were consistent with competitive and reversible inhibition of VEGF-R2/KDR kinase activity in vitro.

Effects of CEP-5214 on Angiogenesis in in Vitro and ex Vivo Bioassays

On the basis of its potent and selective inhibition of the VEGF-R kinases and pronounced inhibition of VEGF-R2 phosphorylation in human and rodent endothelial cells, the activity of CEP-5214 was evaluated in in vitro and ex vivo bioassays of angiogenesis. The rat aortic ring explant model in three-dimensional collagen gel matrices and the HUVEC capillary tube formation assay on a Matrigel synthetic basement membrane matrix are two widely used ex vivo and in vitro systems to model effectively the distinct temporal and spatial events underlying angiogenesis in vivo (52–53). Both assays are sensitive to the angiogenic effects of VEGF and to the antiangiogenic effects of inhibitors of the VEGF-R, VEGF-R2/KDR (35, 57, 57). CEP-5214 displayed statistically significant dose-related inhibition of complete HUVEC capillary tube formation on a Matrigel synthetic basement membrane matrix in the absence of apparent endothelial cell cytotoxicity based on trypan blue exclusion. VEGF-induced capillary-tube formation was inhibited by 21% (P < 0.01), 75% (P < 0.001), and 89% (P < 0.001) at 40, 100, and 400 nM of CEP-5214, respectively (Fig. 3). The antiangiogenic response to CEP-5214 was further evaluated ex vivo in rat aortic ring explant cultures over a 19-day time course in the absence of exogenous VEGF stimulation. Dose-related inhibitory effects on microvessel growth were observed at 4, 20, 40, 100, 200, and 400 nM CEP-5214.

Table 1 Kinase-inhibitory activities of CEP-5214 and CEP-7055

<table>
<thead>
<tr>
<th>Molecular target</th>
<th>IC₅₀ (nM) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEP-5214</td>
<td>CEP-7055</td>
</tr>
<tr>
<td>VEGF-R1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>VEGF-R3</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>TIE2</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>FLT3</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>FGFR1</td>
<td>412 ± 37</td>
</tr>
<tr>
<td>PDGF-RB</td>
<td>406 ± 76</td>
</tr>
<tr>
<td>Kit</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>TRKA</td>
<td>&gt;3,000</td>
</tr>
<tr>
<td>β₁AR</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>MLK1</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>MLK2</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>MLK3</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>DLK</td>
<td>119 ± 24</td>
</tr>
<tr>
<td>JNK1/β1</td>
<td>448 ± 102</td>
</tr>
<tr>
<td>CHK1</td>
<td>&gt;3,000</td>
</tr>
<tr>
<td>CDS1</td>
<td>&gt;3,000</td>
</tr>
<tr>
<td>CHK1/cyclinB</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Rat brain PKCα (α, β, γ isozymes)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>p38α</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

a PKC, protein kinase C.

b ND, not determined

Fig. 1. Chemical structure of CEP-7055 and CEP-5214.
and 400 nM of CEP-5214 (Fig. 4). Statistically significant effects were observed at 40 nM CEP-5214 at days 7 and 11 with a 65% \( (P < 0.01) \) and 53% \( (P < 0.05) \) inhibition of microvessel growth, respectively, in the absence of apparent cytotoxicity to endothelial cells, fibroblasts, and pericytes in these primary aortic ring explants. Stimulation of rat aortic explant cultures with murine VEGF resulted in a more robust angiogenic response relative to unstimulated cultures, but the growth kinetics of aortic ring explants were comparable with that observed in the absence of exogenous VEGF stimulation. Under these experimental conditions, the administration of CEP-5214 resulted in significant \( (P < 0.05) \) inhibition of microvessel growth at 40 nM, relative to control cultures at day 7 during the peak of microvessel growth.

Pharmacokinetic and Pharmacodynamic Profile of Orally Administered CEP-7055 in Mice

Pharmacokinetic studies in rats and nude mice demonstrated that after p.o. administration of CEP-7055, only CEP-5214 is detected in the plasma compartment; no CEP-7055 is detectable within the shortest time frame (\(~5\) min postdosing) evaluated (data not shown). Moreover, p.o. administration of CEP-7055 produced similar or better overall systemic exposure to CEP-5214 relative to molar-equivalent dosages of CEP-5214 (Fig. 5). Dose-proportional plasma levels of CEP-5214 were observed between the 3.57- and 11.9-mg/kg dose of orally administered CEP-7055. The p.o. bioavailability of CEP-7055 in nude mice (measured as CEP-5214) was \(~15\)%, a value comparable with that observed in CD-1 mice as well. The protein binding of CEP-5214 to mouse plasma \(-1\) acidic glycoprotein (AGP) was 39% at 10 \( \mu M \), as assessed by quinolidine red displacement.

Single-dose p.o. administration of CEP-7055 or CEP-5214 directly to nude mice bearing VEGF-R2 expressing SVR tumor xenografts resulted in a time-dependent inhibition of VEGF-R2/FLK-1 phosphorylation in tumor lysates. Inhibition peaked at \(~30\) min postadministration, was still apparent at 3 h, and returned to baseline levels of receptor phosphorylation by 4 h, results consisted with the plasma profile of CEP-5214 after p.o. CEP-7055 administration in mice (data not shown). Similar observations for the inhibition of VEGF-R2/FLK-1 phosphorylation were obtained after p.o. administration of CEP-7055 or CEP-5214 directly in murine lung tissue lysates from VEGF-stimulated mice.

Collectively, these biochemical efficacy data examining VEGF-R2/FLK-1 phosphorylation profiles \textit{in vivo} confirm the molecular target-directed inhibitory activity of orally administered CEP-7055 or CEP-5214.

Effects of CEP-7055 Administration p.o. in \textit{in Vivo} Angiogenesis Models

Efficacy of p.o. CEP-7055 Administration in the PAEC-VEGF/bFGF-Matrigel Implant Model in Nude Mice. To determine whether p.o. administration of CEP-7055 could inhibit VEGF-medi-
ated angiogenic responses in vivo independent of its potential antitumor activity, we conducted a series of studies in models of normal and pathological angiogenesis similar to those reported previously (60). The effect of p.o. administration of CEP-7055 on neovascularization was examined in the PAEC-VEGF/bFGF-Matrigel implant model in athymic nude mice. As shown in Fig. 6, p.o. administration of a 0.35- to 23.8-mg/kg/dose of CEP-7055 b.i.d. for 8 days resulted in a dose-related inhibition of neovascularization in the absence of apparent toxicity or morbidity, with an 82% inhibition (P < 0.01) relative to vehicle-treated control mice, with a MED for significant antiangiogenic activity of ~7 mg/kg/dose of CEP-7055 p.o. b.i.d. These in vivo findings provided additional corroboration of in vitro and ex vivo data demonstrating potent and significant p.o. antiangiogenic activity of CEP-7055 on the host vasculature.

Efficacy of p.o. CEP-7055 Administration on Inflammation-induced Angiogenesis in Vivo. Given the involvement of VEGF and a number of angiogenic cytokines in inflammatory angiogenesis and leukocyte adhesion to microvessels (66–69), the efficacy of p.o. CEP-7055 administration was evaluated in a murine chronic granulomatous tissue model of chronic inflammation (61) in which effects on both granuloma formation and vascularity (based on carmine red dye content) could be assessed. Administration p.o. b.i.d. of a 23.8-mg/kg/dose of CEP-7055 for 6 days resulted in a 30% reduction in granuloma mass (P < 0.01) relative to vehicle-treated control mice, and a 42% reduction (P < 0.01) in granuloma vascularity. These data indicate that, in addition to its direct antiangiogenic activities, CEP-7055 demonstrates significant anti-inflammatory activity in a murine model of chronic inflammatory angiogenesis, consistent with its inhibition of VEGF-R-mediated activity in vivo.

Effects of p.o. CEP-7055 Administration on VEGF-induced Plasma Extravasation in Rats. The vascular permeability activity of VEGF is one of its characteristic functions that distinguishes it from a number of other angiogenic cytokines (6, 7, 14). To further confirm that p.o. administration of CEP-7055 directly inhibits VEGF-R2/KDR-dependent events in the vasculature, we assessed its ability to attenuate VEGF-induced vascular permeability in rat skin using a modification of the Miles/Evan’s Blue assay. The administration of CEP-7055 at the 23.8-mg/kg/dose p.o. resulted in a significant (P < 0.01) and sustained inhibition of VEGF (3 ng)-induced plasma extravasation (as measured by Evan’s Blue dye release) in the skin of male Sprague Dawley rats over a 6-hour period after a single p.o. dose, compared with vehicle (1% aqueous acetic acid) controls, achieving a maximum 66% inhibition at 1 h postdose (Fig. 7). Basal plasma extravasation (0.9% saline solution without VEGF) was not effected by CEP-7055 administration at any time point evaluated. Similarly, p.o. administration of CEP-7055 resulted in a significant...
Dose-related inhibition of VEGF (3 ng)-induced plasma extravasation in rats when administered 1 h before measurement of local plasma extravasation, with an ED$_{50}$ of 20 mg/kg/dose CEP-7055. Basal plasma extravasation (0.9% saline solution without VEGF) was not effected by CEP-7055 administration at any dose evaluated.

Collectively, these in vivo data in three distinct angiogenesis models demonstrate that p.o. administration of CEP-7055 exhibits potent and significant dose-related inhibition of VEGF-mediated biological processes involving various components of normal and pathological angiogenesis in vivo.

Antitumor Efficacy of Chronic p.o. CEP-7055 Administration on the Growth of Human and Murine s.c. Xenografts in Nude Mice and in the Inhibition of Tumor-associated Angiogenesis. The antitumor efficacy and tolerability of chronic p.o. CEP-7055 administration were investigated in a therapeutic context against a series of established human and rodent tumor xenografts implanted s.c. in athymic nude or syngeneic mice. These data are summarized in Table 2 and illustrated in Figs. 8 and 9. Administration of p.o. b.i.d. CEP-7055 for periods of 10–60 days resulted in dose-related growth inhibition of multiple human and rodent s.c. tumor xenografts ranging from 50–90%, relative to vehicle-treated control mice at doses of 11.9–23.8 mg/kg/dose in 9 of the 10 s.c. tumor xenograft models evaluated, including CALU-6 lung carcinomas; HCT-116, HT-29, and HCT-116/LX.
COLO 205 colon carcinomas; U251MG glioblastomas; and MCF-7 breast carcinoma xenografts (Table 2). The MED for significant antitumor efficacy was observed at \((3.57 \pm 0.26) \times 10^6 \) cells in several s.c. human tumor models, including the A375 melanoma, U87MG glioblastoma, and ASPC-1 pancreatic ductal carcinoma, with partial tumor regressions observed in the U87MG model at doses of CEP-7055 at \((11.9 \pm 0.5) \times 10^6 \) cells (Fig. 8). The plasma concentrations \((C_{\text{max}})\) of CEP-5214 associated with antitumor efficacy in these tumor models (and those described below) in normal or immunocompromised mice were in the range of 50–200 nM.

The effects of initial tumor volume on the responsiveness to p.o. CEP-7055 administration were evaluated in the highly aggressive ASPC-1 pancreatic carcinoma xenograft model. Chronic p.o. b.i.d. administration of CEP-7055 at \((11.9 \pm 0.5) \times 10^6 \) cells to nude mice bearing ASPC-1 pancreatic carcinoma xenografts of various initial tumor volumes \((150 \text{ mm}^3, 400 \text{ mm}^3, \text{ and } 900 \text{ mm}^3)\) for periods of 30–35 days resulted in significant inhibition of tumor xenograft growth over a comparable time course of treatment, independent of initial tumor volume (Fig. 9). No overt toxicity or significant morbidity (body weight loss) was observed on chronic p.o. administration of CEP-7055 in these in vivo studies. Evaluation of gastrointestinal histopathology in mice receiving p.o. CEP-7055 further revealed no significant findings, although mild focal mucosal inflammation with lymphoid Peyer’s patch hyperplasia was associated with p.o. administration of the dosing vehicle in several cases.

**Effects of CEP-7055 on IMD in Vivo and Tumor Cell Proliferation in Vitro.** IMD was evaluated immunohistochemically as an indirect assessment of effects on tumor angiogenesis in several human tumor xenografts from CEP-7055-treated mice and compared with the IMD from corresponding vehicle-treated mice. The percentage reduction in IMD (as assessed by Factor VIII and CD34 immunostaining) in CEP-7055-treated A375 melanoma and U87MG glioblastoma xenografts after 30 days of p.o. administration relative to oral-vehicle-treated control mice was 20–22% (\(P < 0.05\)). Chronic CEP-7055 administration also resulted in a 36% reduction (\(P < 0.05\)) in both the mean IMD (as assessed by CD34 immunostaining) and the mean microvessel density per mm² in pancreatic tumor xenografts relative to vehicle-treated tumors, and a 23% reduction in CD34 immunostaining in orthotopic RENCA tumor pulmonary metastases (data not shown).

Further evidence to suggest that the antitumor efficacy of CEP-7055 is attributable predominantly to its inhibition of VEGF-mediated angiogenic processes was the absence of antiproliferative or proapoptotic activity of the active moiety, CEP-5214, on tumor cells directly in vitro. CEP-5214 had no effect on the proliferation or viability of these tumor cell lines at concentrations up to 3 μM. These findings lend additional support to the absence of direct antitumor activity of this compound at concentrations demonstrating significant and sustained antitumor and antiangiogenic efficacy in vivo.

**Effects of p.o. Administration of CEP-7055 on Primary Tumor Growth and Metastatic Profile in Orthotopic Models of Human Prostate Carcinoma and Murine RENCA.** The antitumor efficacy of CEP-7055 administration on local and distant metastatic tumor growth was also examined in more clinically relevant orthotopic tumor models of hormone-sensitive human prostate carcinoma (in athymic nude mice) and murine RENCA in BALB/c syngeneic mice. In the LNCaP orthotopic prostate carcinoma model, treatment was initiated 1 day after orthotopic implantation of LNCaP cells in male athymic nude mice. Administration of CEP-7055 or vehicle was performed for 21 days, at which time animals were euthanized, and orthotopically grown prostate tumors were evaluated. The take rate for the prostate tumors was 100% (10 of 10 mice) in the vehicle-treated control group. Administration p.o. of CEP-7055 had a significant effect on the growth (tumor wet weight) of orthotopically implanted LNCaP human prostate carcinoma xenografts, resulting in a 52% reduction in prostate tumor wet weight (\(P < 0.05\)) (data not shown).

In a series of experiments in the orthotopic RENCA model, 2 days after recovery from surgery to implant RENCA cells beneath the renal capsule, groups of mice were randomized into treatment groups and received CEP-7055 at \((3.57 \pm 0.26) \times 10^6 \) cells per mouse at 23.8 mg/kg dose p.o. b.i.d. at 23.8 mg/kg dose p.o. b.i.d. or 1% aqueous acetic acid vehicle p.o. b.i.d. (100 μl/dose). The administration of CEP-7055 for 21–26 days had no significant effect on the weight of primary orthotopic tumor-bearing kidneys, despite inhibiting significantly the growth of RENCA s.c. implanted tumor xenografts (Table 2). A significant effect of CEP-7055 administration was observed, however, on reducing the overall metastatic burden (pulmonary and lymph node metastases) in the RENCA model, as manifested in both the severity of metastatic scores obtained and the weights of lung tissue with metastatic nodules (Fig. 10), with values approaching those observed in normal, non-RENCA-
implanted BALB/c mouse lungs of similar body weight (Fig. 10A). Significant reductions in tumor microvessel density (CD34 staining) were also observed in metastatic lesions as noted above. Administration p.o. of CEP-7055 resulted in a larger percentage of mice having a lower overall metastatic score from their orthotopically implanted RENCA, relative to control mice, but did not result in completely eliminating a high metastatic burden in all of the tumor-bearing animals. These results obtained with p.o. CEP-7055 at 23.8 mg/kg/...
Inhibitor of these VEGF receptor subtypes in addition to KDR/VEGF-R2, through which the VEGFs exerts their mitogenic, chemotactic, and vascular permeabilizing effects on the vascular endothelium. We have demonstrated in in vitro and ex vivo biochemical and biological assays that CEP-5214 reversibly inhibits VEGF-induced phosphorylation of all three VEGF-R kinases with a cellular IC_{50} of ~10 nM in both murine and human endothelial cells. Single-dose p.o. or s.c. administration of CEP-7055 to CD-1 mice at 20 mg/kg/dose b.i.d. equivalents resulted in a significant and reversible inhibition of VEGF-R2/FLK-1 phosphorylation in murine SVR tumors or murine lung tissues for 2–3 h postdose and returned to baseline VEGF-R2/FLK-1 phosphorylation by 4 h. Similar types of pharmacodynamic approaches to assess in vivo biochemical efficacy have been reported for the evaluation of orally active VEGF-R kinase inhibitors preclinically, and have demonstrated a comparable time course for the inhibition of VEGF-R2/FLK-1 phosphorylation after single-dose administration in vivo (44).

The p.o. administration of CEP-7055 at doses of 11.9–23.8 mg/kg/dose b.i.d. to normal or immunocompromised mice results in significant and dose-related inhibition of VEGF-mediated neovascularization (PAEC-VEGF/bFGF-Matrigel implants) by up to 82%, relative to controls, and inhibits significantly both vascularity and granuloma formation in a murine model of chronic inflammation-induced angiogenesis, a process in which VEGF has been demonstrated to play a salient role (61, 67–69). In addition to the activity of CEP-7055 in inhibiting VEGF-mediated neovascularization and inflammation-induced angiogenesis, p.o. administration of CEP-7055 results in significant and sustained (>6 h) dose-related inhibition of VEGF-induced dermal vascular permeability in rats (Evan’s dye assay) with an ED_{50} of 20 mg/kg/dose. Earlier studies demonstrated that acute and chronic p.o. administration of CEP-7055 to rats inhibited intravitreal VEGF-induced retinal vascular permeability and leakage significantly (62 and 52%, respectively), and completely inhibited diabetes-induced retinal vascular permeability, although having no effects on basal vascular permeability (70). Collectively, these data indicate that p.o. administration of CEP-7055 at doses of 11.9–23.8 mg/kg/dose b.i.d. inhibits VEGF-mediated signaling and angiogenesis directly in vivo under both physiological and pathological conditions in the absence of toxicity or pronounced morbidity.

The most salient feature of the pharmacological profile of CEP-7055 is the observation that chronic p.o. administration of 3.57–23.8 mg/kg/dose b.i.d. results in dose-related inhibition of s.c. tumor xenograft growth ranging from 50–90%, relative to vehicle-treated mice against a broad range of human and rodent tumors varying in their histological origin, latency and growth rate, and responsiveness to conventional cytotoxic agents. There are a number of influences that may account in part for differences in the range and magnitude of antitumor responses observed on chronic CEP-7055 administration. These influences include the distinct growth profiles of the varied tumor xenografts examined; the role of the tumor microenvironment, i.e., the extent of localized hypoxia and ischemia within a given tumor xenograft, over the time course of treatment on tumor response; the varied dependency of particular tumor types on VEGF’s versus other angiogenic cytokines at specific stages of their vascular growth and maturation; and tumor-specific differences in the temporal expression and/or activation of key intracellular mediators of VEGF-induced angiogenesis, e.g., AKT/phosphatidylinositol 3’-kinase, p44/42 mitogen-activated protein kinase, specific integrins and adherens (70–75).

This tumor inhibition profile was observed in both immunocompromised and immunocompetent mice when given CEP-7055 for periods ranging from 10 to 65 days. In several distinct tumor xenograft models, including the A375 human melanoma, U87MG human glioblastoma, ASPC-1 pancreatic carcinoma, and SVR murine angiosarcoma, the minimum effective p.o. dose for sustained and significant
antitumor efficacy was 3.57 mg/kg/dose b.i.d. In the case of the U87MG model, monotherapy with CEP-7055 at doses of 11.9 mg/kg/dose b.i.d. or above resulted in a 50% incidence of partial tumor regressions (50–90% reduction of initial tumor volume). Partial or complete tumor regressions in select tumor xenograft models have been observed with monotherapy administration of several other VEGF-R kinase inhibitors, including PTK-787 in CWR-22 prostate carcinomas (35), ZD6474 in PC-3 prostate carcinomas (41), and SU11248 (a VEGF-R2/PDGF-RB kinase inhibitor), in the A431 epidermoid and COLO 205 colon carcinoma models (42). Of note is the fact that the administration of CEP-7055 resulted in significant and sustained tumor growth inhibition in ASPC-1 pancreatic carcinoma xenografts, independent of initial tumor volumes, even in tumors of ~1.0 cm³. Similar findings have been reported with ZD6474 in established CALU-6 lung tumor xenografts (40, 41) and could have significant implications for the clinical administration of these antitumor agents.

The extent to which the weaker inhibitory activities of CEP-5214 or its prodrug, CEP-7055, against c-Kit, FGF-R1 kinase and PDGF-Rβ (Table 1) contribute to the in vivo pharmacological profile of CEP-7055 is not clear, although several antiangiogenic agents, currently (CP-547,632; SU11248) and previously (SU6668) under clinical evaluation, possess one or more of these activities (34, 42, 44, 76–78).

The antitumor efficacy of chronic p.o. administration of CEP-7055 was not confined to s.c. tumor xenografted tumors but extended to orthotopically implanted human prostatic carcinomas (60) and murine RENCA xenografts in both immunocompetent and immunocompromised mice. This renal carcinoma model has been reported to be sensitive to the administration of several antiangiogenic agents, including TNP-470 (64) and, in particular, PTK-787 (35–37). Curiously, although CEP-7055 inhibited the extent of metastases in the RENCA model, as well as the growth of s.c. implanted RENCA tumors, marginal antitumor activity was observed against primary RENCA tumor growth within the renal capsule. Similar findings for more pronounced activity of antiangiogenic agents (e.g., PTK-787) against metastases versus primary tumor growth in the RENCA model have been reported (35). The homogeneous and extensive vascularization throughout these tumors suggests that, in rapidly growing RENCA tumors, the tumor cell proliferation rate is superior to that of tumor-associated endothelial cells and results in partial angiogenesis-independent tumor growth (36). This fact, and the proximity of orthotopically implanted RENCA tumors to an abundant vascular supply in the kidney, could account for the marginal effect of CEP-7055 on primary RENCA tumor growth, despite its significant effects on the seeding and subsequent growth of distant lung metastases and on the inhibiting of s.c. implanted RENCA xenograft growth, processes that would be predicted to be more angiogenesis-dependent. An additional explanation for the significant antimetastatic efficacy of...
CEP-7055: THE PRODRUG OF CEP-5214


19. Stacker, S. A., Achen, M. G., Jussila, L., Baldwin, M. E., and Alitalo, K. Lymphatic metastases in multiple solid tumor types observed at total daily p.o. doses of 50–100 mg/kg/dose (PTK-787; Ref. 35–38), 25–100 mg/kg/dose (ZD 6474; Refs. 40, 41), 50 mg/kg/dose (CP-547,632; Ref. 44), and 40 mg/kg/dose (SU11248; Ref. 42). This broad antitumor efficacy profile of CEP-7055, the observed reductions in IMD (20–36% relative to control tumors depending on tumor type) on its chronic administration in several tumor xenograft models, and the significant in vivo efficacy observed with chronic CEP-7055 administration in a variety of VEGF-mediated angiogenesis models described in this report, are consistent with primarily indirect, i.e., antiangiogenesis-related, antitumor effects of this pan VEGF-R kinase inhibitor rather than with a direct proapoptotic effect on individual tumor cells per se. In support of this conclusion, is the fact that 3–10 μM concentrations of CEP-5214 were required to achieve significant antiproliferative or proapoptotic effects against a panel of human tumor cell lines in vitro, significantly higher than the plasma concentrations (50–200 nm) at which in vivo efficacy has been observed with CEP-7055 administration in preclinical murine models.

In summary, we have identified and are developing CEP-7055, the prodrug of CEP-5214, an orally active, low-nanomolar pan VEGF-R kinase inhibitor that demonstrates significant and durable antiangiogenic efficacy in vitro, ex vivo, and in vivo, and antitumor efficacy against s.c. and orthotopically implanted human and rodent tumors in vivo. The pharmacological, pharmacokinetic, and tolerability profile of this agent in preclinical studies is compatible with chronic administration against a variety of disease states in which VEGF-mediated angiogenesis plays a salient role. CEP-7055 is currently in Phase I clinical trials as an orally administered therapy in patients with a variety of solid tumors.

REFERENCES


5347, 2002.


Announcements

(Requests for announcements must be received at least three months before publication.)

FUTURE ANNUAL MEETINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

2004 March 27–31, Orlando, FL
2005 April 16–20, Anaheim, CA

AACR SPECIAL CONFERENCES IN CANCER RESEARCH

A number of meetings are now being organized in the AACR’s series of smaller scientific meetings. Following are the topics, dates, locations, and program committees for these meetings. When full details of each meeting are available, AACR members will be the first to receive complete brochures and application forms for participation in these important conferences. Nonmembers may receive this information by sending their names and addresses to Meetings Mailing List, American Association for Cancer Research, 615 Chestnut Street, 17th Floor, Philadelphia, PA 19106-4404. Up-to-date program information is also available via the Internet at the AACR’s website (http://www.aacr.org).

AACR-NCI-EORTC INTERNATIONAL CONFERENCE ON MOLECULAR TARGETS AND CANCER THERAPEUTICS

November 17–21, 2003
Hynes Center, Boston, MA

Chairpersons
Charles L. Sawyers, Los Angeles, CA
Edward A. Sausville, Bethesda, MD
Jaap Verweij, Rotterdam, The Netherlands

SIXTH JOINT CONFERENCE OF THE AACR AND JCA, ADVANCES IN CANCER RESEARCH

January 25–29, 2004
Hilton Wai Koloa Village, Wai Koloa, Hawaii

Chairpersons
Waun Ki Hong, Houston, TX
Takahashi Tsuruo, Tokyo, Japan

CALENDAR OF EVENTS


10th Hong Kong International Cancer Congress, November 19–21, 2003, Faculty of Medicine Building, The University of Hong Kong, Hong Kong. Contact: 10th HKICC Congress Secretariat, Department of Surgery, University of Hong Kong Medical Centre, Queen Mary Hospital, Hong Kong. Phone: 852.2818.0232 or 852.2855.4235; Fax: 852.2818.1186; E-mail: mededcon@hku.hk; Website: www.hkicc.org.

Third International Conference and 9th Annual Meeting of the International Society of Cancer Chemoprevention (ISCaC): Controversies in Tumor Prevention and Genetics, February 12–14, 2004, University of St. Gallen, Switzerland. E-mail: info@oncoconferences.ch; Website: www.oncoconferences.ch.


11th Conference on Advances in Neuroblastoma Research, June 16–19, 2004, Genoa, Italy. E-mail: anr2004@neuroblastoma.org; Website: www.anr2004.org.

6th International Conference on Head and Neck Cancer, August 7–11, 2004, Marriott Wardman Park, Washington, DC. Contact: Concepts in Meeting & Events, 1805 Ardmore Boulevard, Pittsburgh, PA 15221. Phone: 412.243.5156; Fax: 412.243.5160; E-mail: ssteighnercme@aol.com.

Molecular Targets for Cancer Therapy: 3rd Biennial Meeting, October 1–5, 2004, Don Cesar Beach Resort & Spa, St. Petersburg Beach, FL. Contact: Ann Gordon. Phone: 813.903.4975; E-mail: gordonac@moffitt.usf.edu.
Corrections

In the article by T. Wissniowski et al., titled “Activation of Tumor-specific T Lymphocytes by Radio-Frequency Ablation of the VX2 Hepatoma in Rabbits,” which appeared in the October 1, 2003 issue of Cancer Research (pp. 6496–6500), the names of the first two authors were misspelled. The correct author list is as follows: Thaddäus Till Wissniowski, Johannes Hänslker, Daniel Neureiter, Markus Frieser, Stefan Schaber, Birgit Esslinger, Reinhard Voll, Deike Strobel, Eckhart Georg Hahn, and Detlef Schuppan.

In the article by B. Ruggeri et al., titled “CEP-7055: A Novel, Orally Active Pan Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases with Potent Antiangiogenic Activity and Antitumor Efficacy in Preclinical Models,” which appeared in the September 15, 2003 issue of Cancer Research (pp. 5978–5991), two co-authors, Shi Yang (Department of Biochemistry, Cephalon, Inc.) and Sonya Pritchard (Department of Oncology, Cephalon, Inc.), were inadvertently omitted from the list of authors. The correct list is as follows: Bruce Ruggeri, Jasbir Singh, Diane Gingrich, Thelma Angeles, Mark Albom, Shi Yang, Hong Chang, Candy Robinson, Kathryn Hunter, Pawel Dobrznanski, Susan Jones-Bolin, Sonya Pritchard, Lisa Aimone, Andres Klein-Szanto, Jean-Marc Herbert, Francoise Bono, Paul Schaeffer, Pierre Casellas, Bernard Bourie, Roberto Pili, John Isacs, Mark Ator, Robert Hudkins, Jeffrey Vaught, John Mallamo, and Craig Dionne.

In the article by Y-T Tai et al., titled “Insulin-like Growth Factor-1 Induces Adhesion and Migration in Human Multiple Myeloma Cells via Activation of β1-Integrin and Phosphatidylinositol 3’-Kinase/AKT Signaling,” which appeared in the September 15, 2003 issue of Cancer Research (pp. 5850–5858), the Western blotting in the bottom “IGF-1R” panel of figure 3B is incorrect. The correct figure appears below:

Fig. 3. IGF-1 stimulates association of IGF-IR and β1 integrin, as well as activation of PI3-K/AKT and ERK pathways. A. serum-starved MM.1S or OPM6 cells were treated with IGF-I for the indicated times. Cell lysate (500 μg) was immunoprecipitated with anti-IGF-IR and anti-IRS-1 Abs and then immunoblotted with anti-pTyr mAb. Membranes were stripped and reprobed with anti-IGF-IR, anti-β1 integrin, and anti-p85 PI3-K Abs. A total of 60 μg of each lysate was also resolved by 8% SDS-PAGE, and immunoblotted with anti-pAKT and anti-pERK Abs. β-tubulin serves as loading controls. B, serum-starved cells were incubated with IGF-I (100 ng/ml) or PMA (100 nM) for 10 min or pretreated with αR3 (5 μg/ml) for 30 min before incubation with IGF-I (100 ng/ml) for 10 min. One mg of cell lysates was immunoprecipitated with 4 μg of anti-β1 integrin Abs. Immunoprecipitates were analyzed by immunoblotting with anti-IGF-IR and anti-β1 integrin Abs.
In the article by G. Yousef et al., titled “Human Kallikrein 5: A Potential Novel Serum Biomarker for Breast and Ovarian Cancer,” which appeared in the July 15, 2003 issue of Cancer Research (pp. 3958–3965), figure 4 was printed incorrectly. Below is the correct figure.

Fig. 4. Fractionation of three biological fluids (serum, ascites fluid from an ovarian cancer patient, and breast milk) by size-exclusion liquid chromatography. The elution profile of molecular mass standards is denoted by arrows. In serum, hK5 elutes as two immunoreactive peaks, one with an apparent molecular mass of 50 kDa (fractions 37–39) and one with an apparent molecular mass of approximately 150–180 kDa (fractions 31–33). The elution profile of another kallikrein with a similar theoretical molecular mass, hK6, is also shown by dashed lines. This kallikrein elutes at a molecular mass of ~35 kDa, corresponding to free hK6. In ascites fluid, the same comments apply as for serum. In breast milk, hK5 elutes mainly as a single immunoreactive peak. hK6 elutes as two distinct peaks, one at a molecular mass of ~35 kDa and another one at a molecular mass of ~100 kDa.
CEP-7055: A Novel, Orally Active Pan Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases with Potent Antiangiogenic Activity and Antitumor Efficacy in Preclinical Models

Bruce Ruggeri, Jasbir Singh, Diane Gingrich, et al.


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