A Selective and Oral Small Molecule Inhibitor of Vascular Epithelial Growth Factor Receptor (VEGFR)-2 and VEGFR-1 Inhibits Neovascularization and Vascular Permeability

NEELA PATEL, LI SUN, DEBORAH MOSHINSKY, HUI CHEN, KATHLEEN M. LEAHY, PHUONG LE, KATHERINE G. MOSS, XUEYAN WANG, AUDIE RICE, DANNY TAM, A. DOUGLAS LAIRD, XIAOMING YU, QINGLING ZHANG, CHO TANG, GERALD MCMAHON, and ANTHONY HOWLETT


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ABSTRACT

Vascular endothelial growth factor (VEGF) is a key driver of the neovascularization and vascular permeability that leads to the loss of visual acuity in diabetic retinopathy and neovascular age-related macular degeneration. Our aim was to identify an orally active, selective small molecule kinase inhibitor of vascular endothelial growth factor receptor (VEGFR)-2 with activity against both VEGF-induced angiogenesis and vascular permeability. We used a biochemical assay to identify 3-[5-methyl-2-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrol-3-yl]-proprionic acid (SU10944), a pyrrole indolinone, which is a potent ATP-competitive inhibitor of VEGFR-2 (Kᵢ of 21 ± 5 nM). In cellular assays, SU10944 inhibited VEGF-induced receptor autophosphorylation (IC₅₀ of 227 ± 80 nM) as well as downstream signaling (IC₅₀ of 102 ± 27 nM). In biochemical assays, SU10944 exhibits potent inhibitory activity against VEGFR-1; weak activity against other related subgroup members, including stem cell factor receptor (SCFR), platelet-derived growth factor receptor β (PDGFRβ), and fibroblast growth factor receptor-1 (FGFR-1); and no detectable activity against other protein tyrosine kinases such as epidermal growth factor receptor (EGFR), Src, and hepatocyte growth factor receptor. In cellular assays, the selectivity for SU10944 to inhibit VEGFR is maintained compared with other tyrosine kinases (IC₅₀ for SCFR of 1.6 ± 0.3 μM, for PDGFRβ of 30.6 ± 13.3 μM, for FGFR-1 of >50 μM, and for EGFR of >50 μM). Upon oral administration, SU10944 gave a clear dose response in the corneal micropocket model with an ED₅₀ value for inhibition of neovascularization of ~30 mg/kg and a maximum inhibition of 95% at 300 mg/kg. Similarly, upon oral administration in the Miles assay, SU10944 potently inhibited VEGF-induced vascular permeability. Our data indicate that small molecule inhibitors of VEGFR signaling have the potential to ameliorate VEGF-induced neovascularization as well as vascular permeability.

In diabetic retinopathy (DR) and exudative age-related macular degeneration (AMD), vascular endothelial growth factor (VEGF) is a driver of both the neovascularization and retinal vascular permeability, which underlie the loss of visual acuity. The temporal and spatial expression patterns of VEGF in these diseases implicate VEGF in ocular neovascularization. VEGF expression levels and activity are elevated in vitreous samples from diabetic patients with active proliferative retinopathy, compared with individuals with nonproliferative diabetic retinopathy, quiescent proliferative retinopathy, nondiabetic individuals, or individuals with nonischemic ocular disease (Adamis et al., 1994; Aiello et al., 1994). The levels of VEGF are positively correlated to the clinically observed degree and stage of retinal neovascularization (Aiello et al., 1994). In AMD, VEGF is expressed in choroidal neovascular membranes (Frank et al., 1996).

As with ocular neovascularization, elevated levels of VEGF are associated clinically with ocular edema (Vinore et al., 1995; Funatsu et al., 2002). In preclinical models, VEGF is likewise associated with changes in vascular integrity. Increases in ocular VEGF in diabetic animals correlate with
elevated vascular permeability, before observable retinal proliferative changes (Sone et al., 1997; Gilbert et al., 1998). Local delivery of VEGF by intravitreal implants result in significant increases in retinal permeability by day 3, whereas retinal neovascularization is observed only after 14 days (Alkacem et al., 2000). A similar breakdown of the blood-retinal barrier occurs in primates administered VEGF by intravitreal implant (Ozaki et al., 1997).

Intervention in VEGF signaling, either by decreasing local concentrations of ligand with antisense oligodeoxynucleotides (Robinson et al., 1996) or soluble chimeric receptors (Aiello et al., 1995), or by inhibiting receptor signaling by small molecules (Ozaki et al., 2000), decreases ocular neovascularization, thus confirming the central role of VEGF in this process. Neovascularization in DR and AMD may differ significantly from angiogenesis in other pathological contexts such as tumor angiogenesis where multiple targets have been implicated, including PDGF, FGF, and IL-8 (Laird et al., 2000; Rofstad and Halsor, 2000; Shaheen et al., 2001). Current data strongly suggest that in diabetic retinopathy and exudative age-related macular degeneration, VEGF is the key driver. In a preclinical model of diabetic retinopathy, hypoxia-driven retinal neovascularization, VEGF inhibitors are efficacious but PDGFR inhibitors are not (Ozaki et al., 2001). Similarly in a model of AMD, injury-induced choroidal neovascularization, a VEGF inhibitor decreases choroidal neovascularization ~85%, but administration of a PDGF inhibitor does not decrease choroidal neovascularization (Kwak et al., 2000). The contribution of FGF to neovascularization is less well understood. Although bFGF is expressed in neovascular membranes from AMD and DR patients (Frank et al., 1996) and intravitreally administered FGF has a synergistic effect with VEGF in producing retinal hemorrhage (Wong et al., 2001), retinal neovascularization occurs upon hypoxic challenge even in the absence of bFGF (Ozaki et al., 1998). Moreover, overexpression of bFGF in a transgenic mouse does not produce retinal neovascularization nor increase the degree of neovascularization upon hypoxia (Ozaki et al., 1998).

Inhibition of PDGF could have deleterious effects, particularly in the context of DR. PDGF seems to play a special role in retinal vasculature. Recent reports suggest that PDGF signaling is important for survival of retinal vasculature, specifically pericytes, under conditions of hypoxic or metabolic stress (Kodama et al., 2001; Hammes et al., 2002). In vivo, PDGF plays a role in retinal capillary coverage: PDGF-B-deficient mice (PDGFB +/−) have fewer retinal pericytes and more acellular retinal capillaries than wild-type controls, differences that are more pronounced in diabetic animals. In the hypoxia-induced model of neovascularization, new vessels are twice as prevalent in PDGFB +/− mice compared with wild-type animals (Hammes et al., 2002), suggesting that pericyte deficiency renders endothelial cells more susceptible to angiogenesis. Therefore, a potential side effect of PDGF inhibition could be to accelerate the loss of pericytes, which are implicated in the destabilization of blood vessels during the early stages of diabetic retinopathy. Therefore, given the lack of data supporting a role for PDGF in retinal and choroidal neovascularization as well as the risk of increasing pericyte dropout, a VEGF-selective inhibitor is likely the best choice for treatment of retinopathies.

Our goal was to identify and characterize an orally available, selective VEGFR inhibitor, because angiogenesis and increased vascular permeability in DR and exudative AMD are primarily or solely driven by VEGF in these settings. A selective compound should minimize the potential toxicities resulting from the inhibition of additional kinases and be more likely to give a sufficient therapeutic index for the treatment of nonlife-threatening disease.

Materials and Methods

Chemicals. SU10944 was synthesized as described in patent WO00/08202. Its chemical structure is shown in Fig. 1. Stock solutions of SU10944 were made in dimethyl sulfoxide and stored at −20°C. Dilutions for assays were made fresh before use.

Kinetic Analysis. The catalytic portion of mouse VEGFR-2 was expressed as a glutathione S-transferase (GST) fusion protein after infection of Spodoptera frugiperda (sf9) cells with engineered baculoviruses by standard methods (King and Possee, 1992). GST-VEGFR-2 was purified to homogeneity from infected sf9 cell lysates by glutathione-Sepharose chromatography (Smith and Johnson, 1988). GST-fusion preparations were analyzed by gel electrophoresis and determined to be of high purity, with no detectable breakdown products as determined using protein staining and Western blot analysis for the GST moiety. Protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) using a standard curve with bovine serum albumin (BSA). For the SU10944 Kᵦ determination against VEGFR-2, biochemical kinase reactions were performed as described below (see “Biochemical Assays”) in the presence of various ATP and inhibitor concentrations. Rates were expressed as the increase in time-resolved fluorescence resonance energy transfer (TR-FRET) counts per minute of reaction within the linear reaction time. Kᵦ values were determined by graphical analysis of the plot of the slopes from the double reciprocal plot versus inhibitor concentration. The final Kᵦ value represents the average ± the standard deviation from five independent experiments.

Biochemical Assays. VEGFR-2 and PDGFRβ TR-FRET autophosphorylation assays were performed as described previously (Moshinsky et al., 2003). Briefly, 1 nM VEGFR-2 or 5 nM PDGFRβ was added to a reaction buffer composed of 50 mM HEPES, pH 7.4, 1 mM MnCl₂, 0.01% BSA, and 1 mM dithiothreitol, containing twice the apparent Kᵦ concentration of both ATP and N-terminal biotinylated peptide (KY-tide: KYKKYKKYKKKKKYKYK) in a 50-µl total volume. Reactions were allowed to proceed within the linear reaction time then terminated by the addition of 20 µl of 90 mM EDTA. Eu-W1024-labeled anti-phosphotyrosine PY20 and Streptavidin: SureLight-Allophycocyanin (PerkinElmer Life Sciences, Foster City, CA) were diluted in Tris-buffered saline containing 0.02% BSA and 0.1% Tween 20 and added to a final concentration of 0.5 and 1.6 nM, respectively. After incubation for at least 10 min, samples were
excited at 340 nM, and emissions were read at 665 nM using an LJI
Analyst (LJI Biosystems, Sunnyvale, CA). The increase in signal
for both assays was determined to be time-dependent with a require-
ment for ATP, peptide, kinase, and divalent metal cations. FGFR-1
and EGFR autophosphorylation reactions were performed using im-
munocaptured kinases as described previously (Laird et al., 2000).

The HGFR and VEGFR-1 assays were performed using poly(Glu,
Tyr) 4:1 as a substrate as described previously (Blake et al., 2000).

For the cdk2/cyclin A assay, a scintillation proximity assay method
was used (Amersham Pharmaceutical Assays Development Group,
1995). The SCFR, src, LCK/YES-related Novel tyrosine kinase, and
fn assays were performed in standard TR-FRET format (Kolb et al.,
1998) using peptide substrates found by screening an internal pep-
tide substrate library. Conditions for compound testing were satu-
rating peptide concentration and an ATP concentration of 2 - Km.

**VEGFR-2 Cell Autophosphorylation Assay.** Full-length mouse
VEGFR-2 was cloned into the C-terminal 3xFLAG-tag expres-
sion vector p3xFLAG-CMV-14 (Sigma-Aldrich, St. Louis, MO). Hu-
muman embryonic kidney 293T cells were grown in Dulbecco’s modified
Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum
and grown in a 37°C humidified incubator with 5% CO2. The con-
struct was transfected into 293T cells using LipofectAMINE2000
(Invitrogen, Carlsbad, CA) under manufacturer’s recommendations.
After transfection, cells were starved in DMEM containing no serum
and 0.1% BSA for 24 h. Cells were then split into 96-well plates and
treated with compound in a final concentration of 1% dimethyl
sulfoxide for 2 h. Cells were lysed by the addition of HNTG (50 mM
HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 10% glycerol, 1%
Triton X-100, 1 mM EGTA), and lysates were transferred to polysty-
rene 96-well plates that had been precoated with 1 µg/well of M2
anti-FLAG monoclonal antibody (Sigma-Aldrich) capture the
3xFLAG-tagged kinase. Quantitation of phosphorylation was per-
formed by incubating with horseradish peroxidase-labeled anti-phos-
photyrosine PY99 (Santa Cruz Biotechnology Inc., Santa Cruz, CA),
formed by incubating with horseradish peroxidase-labeled anti-phos-
photyrosine PY99 (Santa Cruz Biotechnology Inc., Santa Cruz, CA),
followed by detection with a 2.2’-azino-di-(3-ethylbenzthiazoline sul-
fonate (6)) diammnonium salt color readout.

**VEGFR-2 Phosphorylation Detected by Western Blot.** NIH/
3T3 cells stably expressing VEGFR-2 were grown to confluence in
DMEM with 10% heat-inactivated calf serum and then incubated
in serum-free medium containing different concentrations of SU10944
for 20 h. After stimulation with human recombinant VEGF165 (R &
D Systems, Minneapolis, MN) at 50 ng/ml for 10 min, cells were lysed
in lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10% gly-
cerol, 0.5% Triton X-100, 100 mM phenylmethylsulfonyl fluoride, 1
mM sodium orthovanadate, and 2 µg/ml leupeptin and aprotinin.
VEGFR-2 protein was isolated with a monoclonal anti-mouse
VEGFR-2 antibody made at Sugen, Inc. (Santa Barbara, CA),
designated L4. Phosphorylation of VEGFR-2 was then analyzed by
SDS-polyacrylamide gel electrophoresis followed by Western blotting
using biotin-labeled anti-phosphotyrosine PY99 (Santa Cruz Bi-
technology, Inc., Santa Cruz, CA), followed by detection with a 2.2’-azino-di-(3-ethylbenzthiazoline sul-
fonate (6)) diammnonium salt color readout.

**VEGFR-2 Cellular Functional Assay.** The assay is an immu-
nounassay for the quantitative detection of human tissue factor
KhUVECs were seeded at 50,000 cell/well in growth medium (endo-
theial cell basal medium; BioWhittaker, Walkersville, MD) 10% fetal
bovine serum and complete supplements containing human
epidermal growth factor (0.5 mL/500 mL), hydrocortisone (0.5 mL/500
mL), gentamicin sulfate amphotericin-B (0.5 mL/500 mL), and bovine
brain extract (2 mL/500 mL) (BioWhittaker) in 24-well plates coated
with 0.2% gelatin. Cells were grown for 2 days to >90% confluence
and then treated with SU10944 and human recombinant VEGF165
(R & D Systems) at 50 ng/mL or LPA (porbrol 12-myristate 13-
acetate; Sigma-Aldrich) at 10 nM for 4 h; negative control cells
received media only. Cells were lysed with HNTG lysis buffer plus
EDTA (1:100), protease inhibitor (1:100), and PMSF (1:100) in 125
µL/well for 20 min at 4°C. Cell lysates were processed using an
IMUBIND tissue factor ELISA kit (American Diagnostics, Green-
wich, CT). Briefly, lysates were transferred to 96-well plate pre-
coated micro-test strips and incubated at 4°C overnight. The micro-
test strips were washed four times with wash buffer, and 100 µL of
biotinylated anti-human tissue factor antibody was added to each
well and incubated for 1 h at room temperature. After washing four
times, 100 µL of diluted streptavidin-horseradish peroxidase-conju-
gated antibody was added to each well, and incubated for 1 h at room
temperature. The wells were washed four times with wash buffer
and incubated with 100 µL of tetramethyl-benzidine (Sigma-Aldrich)
substrate solution for 20 min at room temperature. The reaction
was stopped by the addition of 50 µL of H2SO4 solution, and absorbance
was read on a microplate reader at a wavelength of 450 nm.

**Functional Cellular Assays for SCFR, EGFR, PDGFRB, and
FGFR-1.** For EGFR, PDGFRB, and FGFR-1, the functional activity
of the receptor was measured by a ligand-induced BrdU incorpora-
tion assay in 3T3 cells that endogenously express both FGFR-1 and
PDGFRB but not EGFR. A single cell line stably transfected with
EGFR was used for all assays. Cells seeded in a 96-well plate were
made quiescent by serum deprivation for 24 h and then stimulated
with 1.5 nM VEGF (5.8 nM PDGF (3.8 nM) in the absence or presence of the indicated concentrations
of SU10944 for 20 h. BrdU was added for a 2-h labeling period, and the
cells were fixed. The amount of BrdU incorporation was determined
with an anti-BrdU-peroxidase-conjugated antibody using an ELISA
kit (Roche Diagnostics, Indianapolis, IN). Cell viability after expo-
sure to compounds in the assay format was assessed by substituting
the addition of BrdU with resazurin (1 mg/mL) at 1:100 after the 20-h
incubation. After a 3-h incubation, the absorbance of the samples are
measured at 630 nm in “dual wavelength” mode with a filter reading
at 450 nm, as a reference wavelength, on a Dynatech ELISA plate
reader. Compound was added to wells in the dilutions used to deter-
mine IC50 values, including a negative control in which all compo-
ents were included except for cells and a positive control in which all
components except compound were added. The resazurin assay is
based upon the conversion of the dark blue resazurin dye to a pink
dye in proportion to the metabolic activity of the cells (O’Brien et
al., 2000). For each IC50 value, the standard deviation is reported.

The functional activity of SCF was assessed using a SCF-depen-
dent cell proliferation/survival assay. M07e cells were grown and
expanded in RPMI 1640 medium with 10% fetal bovine serum in the
presence of IL-3 (10 ng/mL) and granulocyte-macrophage colony-
stimulating factor (10 ng/mL). After counting, cells were pelleted
by centrifugation and washed twice with PBS. Cells were resuspended
in medium containing either IL-3 or SCF (100 ng/mL) and aliquoted
into 96-well plates at 50,000 cells/well along with varying concentra-
tions of SU10944. After incubation for 3 d at 37°C in a humidified
incubator with 5% CO2, live cells were quantified by their ability to
metabolize resazurin as described above.

**Corneal Angiogenesis Model.** An intrastromal pocket was sur-
gically created in one of both corneas of each anesthetized Sprague-
Dawley female rat. A slow release hydrorx/sucralfate pellet contain-
ing 150 ng of human recombinant VEGF165 (PeproTech, Rocky Hill,
NJ) was inserted into the pocket as described factor (4 nM) (Kersey
et al., 1996), the pocket closed to self-seal, and the rats given analgesia
and topical antibiotic ointment applied once to the eye. The rats
recovered from anesthesia on a warming pad and were returned to
their cages. SU10944 was administered daily by gavage in a 1.0-ml
suspension of 0.5% methylcellulose (Sigma-Aldrich), 0.025% Tween
20 (Sigma-Aldrich) to four to six rats per dose group beginning the
day before implant, and continuing the length of the study. Four
days after surgery, the corneas of the reanesthetized rats were
examined under a slit lamp microscope and the neovascular response
was quantified by measuring the average new vessel length (VL), the
corneal radius (r = 2.6 mm), and the contiguous circumferential zone
(CH = clock hours where 1 CH is 30°), and applied to the formula
area (in square millimeters) = (CH/12) x 3.14(r2) – (r - VL)), The
rats were then immediately euthanized. Eyes from rats that developed infection as a result of the surgery were not included in the study. Six to eight eyes were included per dosing group. The neovascular areas of the vehicle and 250-mg/kg dosed rat corneas were dissected from the eye, flat mounted, and photographed at 400X with a digital camera mounted on a microscope. Rat corneas implanted with pellets containing no growth factor (placebo pellets) generated no new blood vessel growth (Leahy et al., 2002). All animal treatment protocols were reviewed by and were in compliance with Pharmacia’s Institutional Animal Care and Use Committee.

Miles Assay for Vascular Permeability. The Miles assay for vascular permeability (Miles and Miles, 1952) was adapted to athymic mice as follows. Mice were given a single oral dose of SU10944 or vehicle alone. Simultaneously or at designated later time points, 100 µl of 0.5% Evan’s blue dye (Sigma-Aldrich) in PBS was administered intravenously via the tail vein. One hour later, mice were injected intradermally (in duplicate sites on their backs) with 400 ng of VEGF (human recombinant VEGF165; R&D Systems) dissolved in 20 µl of PBS or (in adjacent duplicate sites) with PBS alone. After an additional 30 min, VEGF-dependent dye leakage from the vasculature into skin was assessed visually and scored semiquantitatively (100, 50, or 0% inhibition for each spot). Two spots per animal allowed each animal to be scored as representing 100, 75, 50, 25, or 0% inhibition. Low-level background effects from time-matched vehicle-treated groups were subtracted out.

Determination of SU10944 Plasma Levels. Plasma samples (100 µl), SU10944 standard or quality control samples in mouse blank plasma were mixed with acetonitrile (300 µl) containing DL-propranolol hydrochloride (internal standard) in a 96-well polypropylene plate (Orochem Technology, Westmont, IL). The plate was mixed by vortex for 1 min and the samples were centrifuged for 10 min at 4000 rpm. Ten microliters of the supernatant was injected onto the liquid chromatograph tandem mass spectrometry system where separation occurred on a BDS HYPERSIL C18 (5 µm, 100 × 4.6 mm) reverse-phase HPLC column (Keystone Scientific, Foster City, CA). The amount of SU10944 and the internal standard in each mouse plasma sample was quantified based on standard curves generated using known amounts of compound ranged from 5 to 10,000 ng/ml. Standard curve samples and quality control samples of SU010944 were prepared by spiking 10 µl of stock standard solutions with 90 µl of blank plasma.

**Results**

We identified SU10944 (Fig. 1) as a potent inhibitor of VEGFR as part of our efforts to synthesize and characterize indolin-2-ones as inhibitors of class III receptor tyrosine kinases (Yarden and Ullrich, 1988). Previous structure-activity relationship studies revealed that modifications on the indolin-2-one core could generate compounds with different kinase selectivity profiles (Sun et al., 1998, 1999, 2000). Modifications of the core have also been found to have a dramatic impact on the pharmaceutical properties of the compounds in terms of solubility, metabolic stability, permeability, plasma protein binding, and pharmacokinetic properties.

After the identification of SU10944 as an inhibitor of VEGFR-2 in biochemical assays (IC\textsubscript{50} of 96 ± 20 nM), we went on to further assess the biochemical activity of the compound against other kinases, as well as its potential to act as a competitor for ATP. SU10944 exhibited competitive inhibition with respect to ATP for VEGFR-2. This is indicated by the fact that the lines from the double reciprocal plot (Fig. 2) converge on the y-axis. The \( K_i \) value was determined to be 21 ± 5 nM. In a panel of kinase assays, SU10944 potently inhibited VEGFR-2 with an IC\textsubscript{50} of 96 ± 20 nM and exhibited even greater activity against VEGFR-1 with an IC\textsubscript{50} of 6 ± 1 nM (Fig. 3). It showed some activity against...
other closely related family members, for example, PDGFRβ and FGFR-1 but exhibited significantly less activity against other receptors (Table 1). The compound is not a pan-kinase inhibitor because no discernible inhibition of more distantly related tyrosine kinases was evident, e.g., EGFR and Src.

We then used a panel of three cell-based assays to determine whether the compound could cross the cellular membrane and inhibit VEGFR-2 within cells. In 293T cells transiently transfected with mouse VEGFR-2, SU10944 exhibited an IC₅₀ of 227 ± 80 nM for receptor autophosphorylation as measured by ELISA (Fig. 4A). Similarly, in an assay to assess the functional activity of endogenous VEGFR-2 in HUVECs, tissue factor production stimulated by VEGF was inhibited with an IC₅₀ value of 102 ± 27 nM (Fig. 4B). However, SU10944 did not inhibit PMA-stimulated release of tissue factor (Fig. 4B). Final confirmation that the compound inhibits VEGFR-2 receptor phosphorylation was obtained by Western blot analysis of 3T3 cells engineered to express mouse VEGFR-2 and then detected on the blot with an anti-phosphotyrosine antibody. SU10944 inhibited receptor phosphorylation confirming the results of the 293 assay in a different cell type and assay formats.

For kinases against which SU10944 was active in biochemical assays, cellular assays were performed to determine whether the activity was maintained in a more physiological setting (Table 2). Similar to the observations in the panel of biochemical assays, significantly higher concentrations of compound were required to inhibit a closely related subgroup of family members such as FGFR-1, SCFR, and PDGFRβ than was required for the inhibition of VEGFR-2 in functional assays. The functional activities of the FGFR-1, FGFRβ, and EGFR were measured by ligand-induced cell proliferation of 3T3 cells as measured by BrdU incorporation. For SCFR, the activity was measured by the SCF-dependent survival of MO7e cells. SU10944 did not exhibit detectable inhibition of EGFR or FGFR-1 (IC₅₀ values > 50 μM). Furthermore, SU10944 was not cytotoxic: the LD₅₀ value was >50 μM for 3T3 cells.

We then assessed the ability of SU10944 to inhibit angiogenesis and vascular permeability in vivo when administered by the oral route. In the rat corneal micropocket model of angiogenesis, a VEGF pellet is implanted in the cornea to stimulate neovascularization. In this model, the compound significantly decreased the angiogenesis, both the number of vascular sprouts as well as the length of the sprouts (Fig. 5).

![Figure 4](https://example.com/Figure4.jpg)

**Fig. 4.** SU10944 inhibits VEGFR-2 receptor phosphorylation and functional activity in cells. a, 293T cells transiently transfected with mouse VEGFR-2 were exposed to varying concentrations of SU10944 and receptor phosphorylation detected by ELISA. Error bars represent standard deviations from n = 3. b, HUVECs were stimulated with either PMA or VEGF. Production of tissue factor was measured by ELISA. Error bars represent standard deviations from duplicate samples. c, 3T3 cells stably transfected with mouse VEGFR-2 were stimulated with VEGF. Anti-mouse VEGFR-2 antibody was used for immunoprecipitation from cellular lysates. Western analysis of immunoprecipitated proteins was performed using anti-phosphotyrosine antibody or anti-mouse VEGFR-2 antibody.

### TABLE 1

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Mean IC₅₀ μM</th>
<th>IC₅₀ μM</th>
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<tr>
<td>VEGFR-2</td>
<td>0.096 ± 0.020 (n = 10)</td>
<td>&gt;20</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>0.006 ± 0.001 (n = 5)</td>
<td>&gt;20</td>
</tr>
<tr>
<td>FLK1</td>
<td>1.60 ± 0.87 (n = 5)</td>
<td>&gt;20</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>1.00 ± 0.08 (n = 4)</td>
<td>&gt;20</td>
</tr>
<tr>
<td>SCFR</td>
<td>1.58 ± 0.27 (n = 5)</td>
<td>&gt;20</td>
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**Note:** The IC₅₀ values for SU10944 were determined by measuring autophosphorylation or substrate phosphorylation, as specified under Materials and Methods.

### TABLE 2

<table>
<thead>
<tr>
<th>Receptor (Cell Type)</th>
<th>IC₅₀ μM</th>
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<tbody>
<tr>
<td>SCFR&lt;sup&gt;a&lt;/sup&gt; (MO7e)</td>
<td>1.6 ± 0.3 (n = 2)</td>
</tr>
<tr>
<td>PDGFRβ&lt;sup&gt;b&lt;/sup&gt; (3T3)</td>
<td>30.6 ± 13.3 (n = 6)</td>
</tr>
<tr>
<td>EGFR&lt;sup&gt;a&lt;/sup&gt; (3T3)</td>
<td>&gt;50 (n = 3)</td>
</tr>
<tr>
<td>FGFR-1&lt;sup&gt;b&lt;/sup&gt; (3T3)</td>
<td>&gt;50 (n = 3)</td>
</tr>
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<sup>a</sup> Functional activity of SCFR was measured in a survival assay for MO7e cells.
<sup>b</sup> Functional activity was measured by BrdU incorporation in 3T3 cells stably transfected with the receptor.
SU10944 inhibited VEGF-induced vascular permeability in a time- and dose-dependent manner (Fig. 7a). Maximum inhibition was observed at the earliest time point (1 h), with decreasing levels of inhibition over the course of 24 h. At 100 mg/kg, the maximum inhibition of vascular permeability was sustained to 2 h, and a 50% reduction in response was still apparent 24 h postdose. In contrast, at the 30-mg/kg dose maximum inhibition was seen at 1 h and decreased dramatically over 24 h to zero. Plasma concentrations of SU01944 were also determined and correlated with inhibition (Fig. 7b). Based on the total data set that reflects a range of doses and times postadministration of compound, we conclude that SU10944 plasma exposures of 250 ng/ml (844 nM) result in 50% inhibition of VEGF-mediated vascular permeability in vivo.

**Discussion**

We have identified and characterized a new small molecule inhibitor of VEGF signaling that inhibits both angiogenesis and vascular permeability when administered orally. The compound, SU10944, is a potent ATP-competitive inhibitor of the kinase activity of purified VEGFR-2 with a \( K_i \) of 21 ± 5 nM and an IC\(_{50} \) of 96 ± 20 nM. SU10944 also exhibits potent activity against VEGFR-1, with an IC\(_{50} \) of 6 ± 1 nM. The compound maintains activity in cellular assays against both the mouse (flk-1) and human (KDR) forms of VEGFR-2. In the cellular assay for inhibition of VEGFR-2 autophosphorylation, the compound exhibited an IC\(_{50} \) of 227 ± 80 nM, a value in good agreement with the IC\(_{50} \) value obtained in our HUVEC assay for receptor function (IC\(_{50} \) of 102 ± 27 nM). In the functional assay, we demonstrated that the inhibition of tissue factor production resulted from inhibition of VEGFR by showing that PMA-stimulated release of tissue factor was not affected by the compound. The HUVEC data suggest that the compound will not only be functionally active against human VEGFR-2 but should be active in endothelial cells that are the target for inhibition of angiogenesis and vascular permeability. We were further able to confirm the nature of the cellular activity of SU10944 by visualizing inhibition of receptor phosphorylation by Western analysis.

From a panel of biochemical and cellular assays, we conclude that SU10944 is a relatively selective inhibitor with a strong preference for VEGFRs. The kinase activity of both VEGFR-1 and VEGFR-2 are potently inhibited by the compound, with low- to mid-nanomolar IC\(_{50} \) values. Some activity was observed in the biochemical assays, particularly against other members of the class III receptor tyrosine kinases, for example, PDGFR\(\beta \) (1000 ± 83 nM) and SCFR (1580 ± 270 nM). SU10944 exhibited little or no activity against the other kinases surveyed, which represent a range of tyrosine kinases as well as some serine/threonine kinases. We limited our cellular assays to those kinases that had shown activity in biochemical assays, plus one more distantly related kinase. Consistent with the biochemical observations, the compound displayed very limited cross-reactivity in the cellular functional assays. The most notable cross-reactivity occurred against SCFR (IC\(_{50} \) of 1600 ± 300 nM); however, compared with VEGFR functional readout (IC\(_{50} \) of 102 ± 27 nM), there was an 18-fold selectivity. Selectivity at the cellular level was good compared with PDGFR\(\beta \) (IC\(_{50} \) of 30.6 ± 13.3 μM), with a ratio of 340×.
Although SU10944 inhibited the kinase activity of purified VEGFR-2 with an IC_{50} of 96 ± 20 nM, when the compound was tested in cellular assays the apparent IC_{50} value shifted to 227 ± 80 nM in the autophosphorylation assay but was in very close agreement with the functional assay (IC_{50} of 102 ± 27 nM). A more notable discrepancy was observed between the IC_{50} values for the kinase activity of purified PDGFRβ (1,000 ± 83 nM) compared with the cellular assay (IC_{50} of 30.6 ± 13.3 μM). Modest discrepancies between the biochemical and cellular values are not uncommon findings with small molecule enzyme inhibitors because the physical properties of the compounds as well as the assay format become important when translating from biochemical to cellular activity. To inhibit activity of the kinase within a cellular context, the compound must cross the cell membrane and retain activity in the presence of cellular proteins. In addition, differences in assay formats can influence the observed IC_{50} values.

In the context of potential treatments for exudative AMD and diabetic retinopathy, a therapeutic molecule should inhibit both neovascularization as well as increased vascular permeability to be maximally effective. In preclinical models, SU10944 potently inhibited both VEGF-induced angiogenesis and vascular permeability after administration by the oral route. In the corneal micropocket model of angiogenesis, we observed a clear dose response with a maximal inhibition of nearly 100%. Compound levels obtained by oral administration clearly achieved sufficient exposure levels to inhibit the functional activity of the receptor. Similarly, in the Miles assay of vascular permeability, a time- and dose-dependent response was observed. The maximum response was nearly 100%, confirming that in a second species and different model, pharmacologically relevant levels of drug were achieved by oral administration.

We note that there is a discrepancy between the in vitro potency of SU10944 (227 ± 80 nM in the autophosphorylation assay, 102 ± 27 nM in the functional assay) and its in vivo activity as measured in the vascular permeability assay (EC_{50} cut-off estimated to be 833 nM). Although there can be many reasons for such observed differences in the translation from in vitro to in vivo results, the main source in this case is likely to be high plasma protein binding of the compound. Other compounds in the series have been shown to have high protein binding; our data suggest protein binding of >95% (data not shown). The unbound concentration of SU10944 in vivo would therefore be roughly 16 nM, a value in better agreement with our in vitro observations.

To develop the compound for potential use in the treatment of human ocular disease, the efficacy of the compound in more relevant disease models, as well as the potential safety of the compound with systemic administration will require careful investigation. The activity of the compound in human retinal endothelial cells and in more physiologically relevant animal models such as retinal vascular permeability in streptozotocin-induced rats, hypoxia-driven retinal angiogenesis in mouse neonates, and laser injury-induced choroidal neovascular model of AMD should be determined. In addition, the therapeutic index of such compounds will be essential to establish because of potential mechanism-based toxicities with systemically administered VEGFR inhibitors, particularly in the context of diabetic comorbidities. One area of particular concern is whether VEGFR inhibitors will further impair coronary collateral formation in diabetic patients, in response to myocardial ischemia. Collateral vessel development occurs by the process of arteriogenesis, the expansion of existing arterioles, a process that is differentially regulated from angiogenesis. Monocyte migration into the area of ischemia is a key process in the formation of the collateral vessels. Current evidence suggests that impaired collateral vessel formation in diabetic individuals results from a signaling defect downstream of VEGFR-1 in monocytes (Walleenberger, 2001), which normally respond to VEGF-A by increased migration. Because the signaling via VEGFR-1 is already impaired in diabetic patients, it remains to be determined...
whether a VEGFR-1 inhibitor would be additive or neutral for collateral formation, or whether a selective VEGFR-2 inhibitor would be preferred for treatment of diabetic retinopathy.

Unlike VEGFR-2, VEGFR-1 has not been implicated directly in mediating VEGF-induced angiogenesis or vascular permeability. However, inhibition of VEGFR-1 may be beneficial in AMD where macropage infiltration has been suggested to play a role in the etiology of the disease. VEGF produced by hypoxic retinal pigmented epithelial cells has been postulated to act as a chemotactic factor for macrophages, which can then secrete additional VEGF as well as other proangiogenic factors.

We have identified and characterized a novel small molecule inhibitor of VEGFR-2, SU10944. This compound is a potent, ATP-competitive inhibitor of VEGFR-2 biochemical activity and is active in the nanomolar range in cellular assays. SU10944 can be administered in vivo by the oral route and achieves sufficient exposure to inhibit nearly all VEGF-stimulated neovascularization and vascular permeability. A selective, well tolerated VEGFR inhibitor, administered orally or by local delivery, should be of therapeutic benefit in both diabetic retinopathy and exudative age-related macular degeneration. In addition, we believe compounds of this nature will be valuable in delineating the role of VEGF in various forms of pathological angiogenesis where multiple kinases may play contributing roles. Clinical trials will be necessary to show the potential prophylactic or therapeutic utility of these novel and selective VEGFR inhibitors in human eye diseases. The discovery of a multiple VEGFR inhibitors representing a variety of pharmacophores offers the opportunity to generate new molecules with increased potency or improved pharmaceutical properties by rational design based on cores of VEGFR-2.

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References