ABSTRACT

Vascular endothelial growth factor, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) and their cognate receptor tyrosine kinases are strongly implicated in angiogenesis associated with solid tumors. Using rational drug design coupled with traditional screening technologies, we have discovered SU6668, a novel inhibitor of these receptors. Biochemical kinetic studies using isolated Flk-1, FGF receptor 1, and PDGF receptor β kinases revealed that SU6668 has competitive inhibitory properties with respect to ATP. Crystalllographic studies of SU6668 in the catalytic domain of FGF receptor 1 substantiated the adenine mimetic properties of its oxindole core. Molecular modeling of SU6668 in the ATP binding pockets of the Flk-1/KDR and PDGF receptor kinases provided insight to explain the relative potency and selectivity of SU6668 for these receptors. In cellular systems, SU6668 inhibited receptor tyrosine phosphorylation and mitogenesis after stimulation of cells by appropriate ligands. Oral or i.p. administration of SU6668 in athymic mice resulted in significant growth inhibition of a diverse panel of human tumor xenografts of glioma, melanoma, lung, colon, ovarian, and epidermoid origin. Furthermore, intravital multiluminescence videomicroscopy of C6 glioma xenografts in the dorsal skinfold chamber model revealed that SU6668 treatment suppressed tumor angiogenesis. Finally, SU6668 treatment induced striking regression of large established human tumor xenografts. Investigations of SU6668 activity in cancer patients are ongoing in Phase I clinical trials.

INTRODUCTION

The sustained growth of solid tumors is dependent on angiogenesis, the growth of new blood vessels from existing host vasculature (1, 2). Several families of RTKs3 have been implicated in this process. These include the VEGF and angiopoietin receptors (reviewed in Ref.3), which are largely dedicated to angiogenesis, and the FGFRs and PDGFRs (reviewed in Refs. 4 and 5), which are involved in diverse developmental and oncogenic processes.

Evidence for the direct role of VEGF and its receptor, Flk-1/KDR, in angiogenesis has been well documented. The temporal and spatial patterns of expression of VEGF and its receptors, along with the results of targeted mutagenesis, demonstrate that they are required for angiogenesis during development (3). Similarly, the role of ligand and receptor in tumor angiogenesis has been clearly demonstrated using tumor models in rodents, in which disruption of VEGF signaling using anti-VEGF antibodies, soluble VEGF receptors, and regulatable expression constructs can inhibit neovascularization and compromise existing tumor vasculature, resulting in inhibition of tumor growth (reviewed in Ref. 6). Elevated VEGF levels have been correlated with increased microvessel counts and poor prognosis in many human tumor types (reviewed in Ref. 7). Due to its central role in angiogenesis and its modest role in normal adults, VEGF signaling is an attractive therapeutic target. Several VEGF receptor-specific kinase inhibitors have entered clinical trials for the treatment of human cancers. To date, these compounds have shown initial indications of good tolerability, and objective responses have been observed in some patients (8).

FGF and PDGF also play critical roles in angiogenesis, sometimes in concert with VEGF. The prototype FGF family member, FGF2, is a potent mitogen of different cell types including vascular endothelial cells and fibroblasts (9). Although FGF2 knockout mice have no apparent defects related to impaired angiogenesis, FGF2 is clearly an angiogenic factor in vivo (10). Additionally, FGF2 has been reported to be synergistic with VEGF and to induce the expression of VEGF (10). FGF is also a tumor cell mitogen and is expressed, along with its receptors, in a variety of human tumor types (11–16).

PDGF and PDGFRs are expressed in microvascular endothelium in vivo when endothelial cell activation and angiogenesis occur. Moreover, PDGF exerts growth-stimulatory effects on pericytes (17) and fibroblast-like cells (18, 19) that surround endothelial cells. Direct evidence for a role of PDGF-B in vasculogenesis was demonstrated in mice deficient in PDGF-B; these mice lacked microvascular pericytes, which normally form part of the capillary wall and contribute to its stability (20). PDGF has been reported to up-regulate other angiogenic factors such as VEGF; thus, it has been postulated that it may also play an indirect activating role in angiogenesis (21, 22).

PDGF and its receptors have been detected in diverse human cancers (23–30), and PDGFRs are expressed on tumor neovasculature and up-regulated during tumor progression (23). Circulating PDGF has been associated with metastases (31) and higher microvessel counts (32). Again suggesting its direct and indirect roles in angiogenesis, PDGFR has been shown to be expressed on vascular endothelial cells as well as smooth muscle cells in the stroma of tumors (33).

The signaling cascades generated by these three ligands and their respective receptors are complex, directly and indirectly affecting tumor angiogenesis and tumor growth. Given the early promise demonstrated by compounds that inhibit VEGF signaling in the clinic and the knowledge that additional players are important in angiogenesis, we developed a multipotent therapeutic agent that augmented favorable anti-Flk-1/KDR properties with efficacy against other angiogenic signaling molecules. Data presented here demonstrate that SU6668, a small molecule synthetic kinase inhibitor, is a potent inhibitor of the tyrosine kinase activity of Flk-1/KDR, PDGFR, and FGFR; inhibits...
tumor vascularization and growth of tumor xenografts of diverse origin; and induces regression of large established tumors.

MATERIALS AND METHODS

**SU6668 Chemical Synthesis**

SU6668, (Z)-3-[2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrrole-3-yl]-propionic acid (CAS Registry Number 210644-62-5), was prepared using a five-step synthesis from the commercially available 4-(2-methoxycarboxyl-ethyl)-3,5-dimethyl-1H-pyrrrole-2-carboxylic acid benzyl ester (34). Briefly, 4-(2-methoxycarboxyl-ethyl)-3,5-dimethyl-1H-pyrrrole-2-carboxylic acid benzyl was hydrogenated over palladium on carbon to give 4-(2-methoxycarboxyl-ethyl)-3,5-dimethyl-1H-pyrrrole-2-carboxylic acid, followed by decarboxylation to give 3-(2,4-dimethyl-1H-pyrrrole-3-yl)-propionic acid methyl ester. It was then formulated using Vilsmeier reagent and hydrolyzed with sodium hydroxide to give 3-(5-formyl-2,4-dimethyl-1H-pyrrrole-3-yl)-propionic acid. The final step involved condensation of the oxindole and the above aldehyde by aldo-condensation in ethanol in the presence of piperidine to give 3-[2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrrole-3-yl]-propionic acid, SU6668.

**Biochemical Tyrosine Kinase Assays**

Recombinant Protein Production. GST-fusion proteins of FGFR1 (kinase domain) and Flk-1 (cytoplasmic domain) were produced in the baculovirus expression system. For both constructs, pFBG2T was used as the transfer vector. This plasmid contains the GST coding sequence, which was amplified by PCR as a BamHI/BglII fragment and cloned into the BamHI site of pFastBac-1 (Life Technologies, Inc., Rockville, MD). The portion of the FGFR1 cDNA encoding amino acids 459–757 was amplified by PCR as an EcoRI/HindIII fragment and ligated downstream of and in frame with the GST coding sequence in pFBG2T. The portion of Flk-1 cDNA encoding amino acids 812–1346 was amplified by PCR as a NotI/SphI fragment and ligated downstream of and in frame with the GST coding sequence in pFBG2T. Recombinant viruses containing the different recombinant transfer vectors were produced following standard protocols (FastBac manual; Life Technologies, Inc.). For protein production, Sf9 cells were infected following standard procedures (35), and fusion proteins were purified by affinity chromatography on glutathione-Sepharose (Sigma, St. Louis, MO). GST-fusion preparations were determined to be of high quality, with no detectable breakdown products [as determined using Western blot analysis for the GST moiety followed by Poncova S staining (data not shown)].

**trans-Phosphorylation Reactions.** Biochemical tyrosine kinase assays to quantitate the trans-phosphorylation activity of Flk-1 and FGFR1 were performed in 96-well microrater plates precoated (20 μg/well in PBS; incubated overnight at 4°C) with the peptide substrate poly-Glu,Tyr (4:1). Excess protein binding sites were blocked with the addition of 1–5% (w/v) BSA in PBS. Purified GST-FGFR1 (kinase domain) or GST-Flk-1 (cytoplasmic domain) fusion proteins were produced in baculovirus-infected insect cells. GST-FGFR1 and GST-Flk-1 were then added to the microtiter wells in 2× concentration kinase dilution buffer consisting of 100 mM HEPES, 50 mM NaCl, 40 μM NaVO₃, and 0.02% (w/v) BSA. The final enzyme concentration for GST-Flk-1 and GST-FGFR1 was 50 ng/ml. SU6668 was dissolved in DMSO at 100× the final required concentration and diluted 1:25 in H₂O. Twenty-five μl of diluted SU6668 were subsequently added to each reaction well to produce a range of inhibitor concentrations appropriate for each enzyme. The kinase reaction was initiated by the addition of different concentrations of ATP in a solution of MnCl₂ so that the final ATP concentrations spanned the Kₘ for the enzyme, and the final concentration of MnCl₂ was 10 mM. The plates were incubated for 5–15 min at room temperature before stopping the reaction with the addition of EDTA. The plates were then washed three times with TBST.

Rabbit polyclonal antiphosphotyrosine antisera were added to the wells at a 1:10,000 dilution in TBST containing 0.5% (w/v) BSA, 0.025% (w/v) nonfat dry milk, and 100 μM NaVO₃ and incubated for 1 h at 37°C. The plates were then washed three times with TBST, followed by the addition of goat antirabbit antisera conjugated with horseradish peroxidase (1:10,000 dilution in TBST) containing 0.5% (w/v) BSA, 0.025% (w/v) nonfat dry milk, and 100 μM NaVO₃, and incubated for 1 h at 37°C. The plates were then incubated for 1 h at 37°C and then washed three times with TBST. The amount of phosphotyrosine in each well was quantitated as described previously (36) after the addition of 2,4′-azino-di-[3-ethylbenzthiazoline sulfonate] as substrate.

**Autophosphorylation Reactions.** Tyrosine kinase assays to quantitate the autophosphorylation activity of PDGFR or EGFR were performed in a similar manner, except that the wells were precoated (0.5 μg/well in 100 μl of PBS) with PDGFRβ- or EGFR-specific monoclonal antibodies (28D4C10 and SUM01, respectively) to capture the respective kinase from lysates of NIH-3T3 cells engineered to overexpress PDGFRβ or EGFR. The reaction buffer for the autophosphorylation studies consisted of 25 mM Tris, 100 mM NaCl, 10 mM MnCl₂, 0.1% (v/v) Triton X-100, and 0.5 mM DTT.

The linear phase of each assay was determined, and reaction rates were calculated from the linear phase of a series of reactions whose duration spanned the linear period. Assays were highly linear with respect to substrate concentration and time (data not shown). Data were analyzed using the Lineweaver-Burk inverse-reciprocal plot of 1/rate versus 1/ATP concentration. Kᵢ calculations were made using the assumption that in the case of competitive inhibition, Kᵢ is increased by a factor of (1 + [I]/Kᵢ), where [I] is the concentration of inhibitor, and in the case of noncompetitive inhibition, V_max is decreased by a factor of (1 + [I]/Kᵢ).

**X-ray Crystallography and Molecular Modeling**

Crystallographic studies of FGFR1-SU6668 complexes were performed as described previously for a related molecule, SU5402 (37). Expression, purification, and crystallization of FGFR1 were performed as described previously (38). Crystals of unliganded FGFR1 were found to grow in space group C2 with two molecules in the asymmetric unit and unit cell parameters when frozen with dimensions of a = 208.9 Å, b = 57.5 Å, c = 65.7 Å, and β = 107.6 degrees. Unliganded crystals were soaked in 500 μl of stabilizing solution [25% polyethylene glycol 10000, 0.3 M (NH₄)₂SO₄, 0.1 M bis-Tris (pH 6.5), 5% ethylene glycol, and 2% DMSO] containing 2 mM SU6668 at 4°C for 1 week. Data were collected on a Rigaku RU-200 rotating anode (Cu Kα) operating at 50 kV and 100 mA and equipped with double-focusing mirrors and a R-AXIS IIC image plate detector. Crystals were flash-cooled in a dry nitrogen stream at −175°C. Data were processed using DENZO and SCALEPACK (39). Difference Fourier electron density maps were computed using phases calculated from the structure of unliganded FGFR1 (40). The crystallography and NMR system (CNS) software suite (40) was used for simulated annealing and positional/B-factor refinement, and O software suite (41) was used for model building. Bulk solvent and anisotropic B-factor corrections were applied during refinement. The average B-factor is 37.0 Å² for all atoms, 37.1 Å² for protein atoms, and 43.0 Å² for SU6668 atoms.

Homology models for the catalytic domains of Flk-1/KDR and PDGFR were generated using the Modeler program (42), with the FGFR1/SU6668 cocystal structure as a reference. Sequence alignment was based on that of Hanks and Quinn (43), with slight modifications. Docking of SU6668 to Flk-1/KDR and PDGFR was performed manually, based on the FGFR1/SU6668 cocystal structure, followed by simple energy minimization.

**Cellular Assays**

All cell lines were propagated as described previously (44, 45). For cellular tyrosine kinase experiments, parental NIH-3T3 mouse fibroblasts and NIH-3T3 cells overexpressing PDGFRβ or EGFR were used. PDGFRβ and EGFR were highly overexpressed in the engineered NIH-3T3 cell lines relative to untransfected NIH-3T3 cells as assessed by Western blot analysis (data not shown). Cells were seeded (3 × 10⁵ cells/35-mm well) in DMEM containing 10% (v/v) FBS and grown to confluence and then quiesced in DMEM containing 0.1% serum for 2 h before drug treatment. HUVECs (seeded at 2 × 10⁴ cells/cm²) were seeded at 2 × 10⁴ cells/cm² and then quiesced in endothelial cell growth media (containing 12 μg/ml bovine brain extract, 10 μg/ml human epidermal growth factor, 1 μg/ml hydrocortisone, 2% (v/v) FBS, 50 μg/ml gentamicin, and 50 μg/ml amphotericin B in modified MCDB 131) and then quiesced in endothelial cell basal media (modified MCDB 131; Clonetics Corp., Walkersville, MD) and then quiesced in endothelial cell basal media (modified MCDB 131; Clonetics) containing 0.5% FBS for 24 h before drug treatment. All cell lines were incubated with the indicated concentrations of SU6668 for 60 min before ligand stimulation (100 ng/ml) for 10 min. Preparation of cell lysates, separation of cellular proteins (30 μg from NIH-3T3 engineered cells, 100 μg from HUVECs), and immunoblotting with antiphosphotyrosine antibody were performed as described previously (36, 44). To determine receptor protein levels,
membranes were stripped with elution buffer (Pierce, Rockford, IL) and reprobed with a polyclonal antibody directed against either KDR, PDGFRβ, FGFR, or EGFR (all from Santa Cruz Biotechnology, Santa Cruz, CA) followed by donkey antirabbit IgG conjugated to peroxidase (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Immunoreactive proteins were detected using an enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech AB). To measure inhibition of ligand-stimulated mitogenesis, HUVECs were treated with SU6668, followed by the addition of ligand, and processed as described previously (36).

In Vivo Tumor Xenograft Experiments

SUGEN, Inc. has an animal facility that is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures are conducted in accordance with the Institute of Laboratory Animal Resources (NIH) Guide for the Care and Use of Laboratory Animals and with SUGEN Animal Care and Use Committee guidelines. Female athymic mice (BALB/c, nu/nu) were obtained from Charles River Laboratories. Animals were maintained under clean room conditions in sterile Micro-isolator cages (Lab Products) with Alpha-Dri bedding and provided free access to sterile rodent chow and water. They received sterile rodent chow and water ad libitum.

A375, Calu-6, A431, C6, and SF763T tumor cells were obtained and cultured as described previously (44). Colo205 and H460 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS and 2 mM glutamine. SKOV3 cells were obtained from American Type Culture Collection and passed five times through mice to yield SKOV3TP5 cells. These cells were cultured in DMEM supplemented with 10% FBS and 2 mM glutamine. Tumor cells (3–10 × 10⁶ cells/animal) were implanted s.c. into the hind flank of mice on day 0 as described previously (45). Daily treatment with SU6668 or vehicle commenced 1 day after implantation of cells (to test efficacy against newly implanted tumors) or when tumors had reached a predetermined average size (to test efficacy against established tumors). SU6668 was delivered i.p. by bolus injection in DMSO or p.o. by gavage in a cremophor-based vehicle as described in “Materials and Methods.” Values are reported in mean ± SD. Mean values were calculated from the average values in each animal. For analysis of differences between the groups, post hoc unpaired Bonferroni t test was used, followed by one-way ANOVA. Results with P < 0.05 were considered significant, and results with P < 0.01 were considered highly significant.

RESULTS

We have previously reported the synthesis and characterization of a series of 3-substituted indolin-2-ones with potent and selective inhibitory activity toward different RTKs (48). The selectivity of these compounds against particular RTKs depended on the substituents on the indolin-2-one core, especially at the C-3 position. Of special interest, 3-[(substituted pyrrol-2-yl)methylidenyl]indolin-2-ones showed selective inhibitory activity against VEGFR receptor tyrosine autophosphorylation at the cellular level. SU5416 is a potent inhibitor of Flk-1/KDR (46), whereas SU5402 (Fig. 1), another compound from this series, was found to inhibit tyrosine phosphorylation of Flk-1/KDR and PDGFR (37, 48). SU5416 and SU5402 were used as prototype compounds for further modifications to develop an inhibitor active against Flk-1, FGFR, and PDGFR.

Effect of SU6668 on Biochemical Tyrosine Kinase Activity.
The effect of SU6668 on biochemical tyrosine kinase activity was investigated in enzyme kinetic experiments. Data in Table 1 demonstrate that SU6668 was a competitive inhibitor, with regard to ATP, of Flk-1 trans-phosphorylation (K_i = 2.1 μM), FGFR1 trans-phosphorylation (K_i = 1.2 μM), and PDGFR autophosphorylation (K_i = 0.008 μM). The respective ATP K_m values for each kinase are also shown in Table 1; consideration of both of these values suggests that SU6668 has greatest potency against PDGFR autophosphorylation but also strongly inhibits inhibits Flk-1 and FGFR1 trans-phosphorylation. In contrast, SU6668 did not inhibit EGFR kinase activity at concentrations up to 10 μM (data not shown). Moreover, the biochemical IC_50 of SU6668 against the EGFR, insulin-like growth factor I receptor, Met, Src, Lck, Zap70, Abl, and cyclin-dependent kinase 2 are at least 10 μM (data not shown), indicating that SU6668 shows a high level of selectivity against other tyrosine and serine/threonine kinases.

Crystallographic and Modeled Structures of SU6668 in Receptors.
To investigate the structural basis for the observation that SU6668 is more potent against PDGFR than Flk-1/KDR or FGF, the three-dimensional structure of SU6668 co-crystallized within the catalytic domain of FGFR1 was determined. Table 2 shows the crystallographic data collection and refinement statistics used to generate the co-crystal model illustrated in Fig. 2, left panel. This structure was then used to construct homology models of SU6668 bound within the ATP binding domains of Flk-1/KDR and PDGFR. Consistent with the high degree of amino acid homology between the kinase domain of FGFR1 and Flk-1/KDR (62%), the modeled structures of Flk-1/KDR (data not shown) and PDGFR (Fig. 2, right panel) were found to be very similar to the co-crystal structure of FGFR1.

The binding of the oxindole core of SU6668 in the active sites of FGFR1 and PDGFR is similar and comparable to what has been
observed and calculated structure factors, respectively (F₀). 199 water molecules, and one sulfate ion (4569 atoms).

mode would be expected for Flk-1, which also has an asparagine at receptor residue 568 (Fig. 2, left panel, Asn568). A similar binding mode would be expected for Flk-1, which also has an asparagine at receptor residue 568 (Fig. 2, left panel, Asn568). A similar binding mode would be expected for Flk-1, which also has an asparagine at receptor residue 568 (Fig. 2, left panel, Asn568). A similar binding mode would be expected for Flk-1, which also has an asparagine at receptor residue 568 (Fig. 2, left panel, Asn568). A similar binding mode would be expected for Flk-1, which also has an asparagine at receptor residue 568 (Fig. 2, left panel, Asn568). A similar binding mode would be expected for Flk-1, which also has an asparagine at receptor residue 568 (Fig. 2, left panel, Asn568). A similar binding mode would be expected for Flk-1, which also has an asparagine at receptor residue 568 (Fig. 2, left panel, Asn568).

described previously in detail for the binding of SU5402 in FGFR1 (37). In both FGFR1 and PDGFR, as in Flk-1/KDR (data not shown), the oxindole core structure of SU6668 forms hydrogen bonds with the receptor backbone at the hinge region. However, the interactions between the proprionic acid side chain of SU6668 and the receptor backbone differ between the receptors. In the SU6668/FGFR1 co-crystal structure, the proprionic acid side chain can occupy several positions, with the primary one being anchored by the interaction between the terminal carboxylate of the side chain and the asparagine at receptor residue 568 (Fig. 2, left panel, Asn568). A similar binding mode would be expected for Flk-1, which also has an asparagine at receptor residue 568 (Asn-921) at this position. However, this interaction is unlikely when SU6668 is bound in the PDGFR active site because the equivalent residue in PDGFR is an aspartic acid (Fig. 2, right panel, Asp688). Instead, when bound in the active site of PDGFR, the carboxylate of the proprionic acid side chain of SU6668 likely forms a more favorable interaction with the side chain of the arginine at residue 604 (Fig. 2, right panel, Arg604). Thus, from a structural viewpoint, the greater potency of SU6668 against PDGFR can be explained by the more favorable interaction of the proprionic acid side chain with the receptor backbone.

Effect of SU6668 on Cellular Tyrosine Kinase Activity. To confirm the measured biochemical activity of SU6668 in a cell-based assay, tyrosine phosphorylation of receptors after ligand stimulation was determined. HUVECs stimulated by VEGF exhibit an increase in tyrosine phosphorylation of KDR. Treatment of cells with SU6668 inhibited this increase in a dose-dependent manner (Fig. 3A). SU6668 also inhibited PDGFR-stimulated PDGFRβ tyrosine phosphorylation in NIH-3T3 cells overexpressing PDGFRβ at a minimum concentration of 0.03–0.1 μM (Fig. 3B). SU6668 inhibited acidic FGF-induced phosphorylation of the FGFR1 substrate 2 (FRS-2) at concentrations of 10 μM and higher (Fig. 3C). However, SU6668 had no detectable effect on epidermal growth factor-stimulated EGFR tyrosine phosphorylation in NIH-3T3 cells overexpressing EGFR at concentrations of up to 100 μM (Fig. 3D). These cellular data demonstrate that SU6668 inhibits Flk-1/KDR, PDGFR, and FGFR but has no activity against EGFR at the concentrations tested.

Effect of SU6668 on Endothelial Cell Mitogenesis. To determine whether inhibition of purified receptors translated into a biological effect in cells, the ability of SU6668 to modulate VEGF- and acidic FGF-induced mitogenesis of endothelial cells was examined. SU6668...
inhibited VEGF-driven mitogenesis of HUVECs in a dose-dependent manner with a mean IC_{50} of 0.34 ± 0.05 μM (Fig. 4). In comparison, FGF-driven mitogenesis of HUVECs was inhibited with a mean IC_{50} of 9.6 ± 0.4 μM. These data demonstrate that, consistent with the biochemical data, SU6668 inhibits mitogenesis of HUVECs induced by both VEGF and FGF. PDGF did not elicit a mitogenic response in HUVECs; thus, the effect of SU6668 could not be examined in this setting. In contrast, SU6668 did not potently inhibit the proliferation of tumor cells grown in culture (IC_{50} > 15 μM; data not shown).

**Effect of SU6668 on Tumor Xenograft Growth.** Given the potency of SU6668 versus Flk-1/KDR, PDGFR, and FGF in vitro, its antitumor properties were determined. p.o. administered SU6668 induced dose-dependent inhibition of A431 tumor growth in the s.c. xenograft model in athymic mice (Fig. 5). No mortality was observed in any treatment group. SU6668 was also efficacious when administered i.p. or p.o. in additional xenograft models, including A375, Colo205, H460, Calu-6, C6, SF763T, and SKOV3TP5 cells (Table 3). Where tested, for the models in which p.o. data are shown, SU6668 administered i.p. at either 75 or 100 mg/kg exhibited statistically significant efficacy (data not shown). These in vivo data demonstrate that SU6668 readily induced >75% growth inhibition against a broad range of tumor types.

**Effect of SU6668 on Tumor Angiogenesis.** To test the hypothesis that inhibition of angiogenesis contributed to the observed effect on tumor growth, the effect of SU6668 on tumor angiogenesis was assessed by intravital multifluorescence videomicroscopy of C6 glioma xenografts implanted into dorsal skinfold chambers in nude mice. As illustrated in Fig. 6, daily treatment with SU6668 significantly suppressed tumor angiogenesis and vascularization throughout the entire 22-day observation period. The dense network of tumor microvessels in animals treated with vehicle alone (Fig. 6A) contrasts with the isolated microvessels (indicated by arrows) seen in animals treated with SU6668 (Fig. 6B). Compared with controls, tumor vessel density in treated tumors was reduced by 65–95% on days 22 and 10, respectively (Fig. 6C). These results clearly demonstrate that SU6668 inhibits tumor-induced microvascular proliferation in vivo and are consistent with SU6668-directed inhibition of at least two processes, VEGF- and FGF-induced endothelial cell mitogenesis (Fig. 4) and FGF- and PDGF-stimulated proliferation of angiogenesis-promoting host pericytes and fibroblasts.

**Effect of SU6668 on Established Tumor Xenografts.** Finally, given the antiangiogenic and antitumor effects demonstrated above, we challenged established tumors with SU6668. SU6668 treatment was initiated in groups of A431 tumor-bearing mice after tumors had reached average group sizes of approximately 200, 400, and 800 mm³.
SU6668 induced dramatic and uniform regression in all groups, regardless of initial tumor size (Fig. 7A). In 20 of 39 treated animals (approximately half of the animals in all three groups), tumors regressed completely, leaving a vestigial scar. After discontinuation of treatment on day 40, all 20 animals remained tumor free for an additional 133 days (one tumor-free animal died on day 91), with three exceptions showing regrowth (Fig. 7B). Resumption of SU6668 treatment (indicated by arrows) resulted in regression of a large regrown tumor (approximately 900 mm^3; Fig. 7B). This is consistent with observations made in the remaining 19 of 39 animals that had tumors that did not completely regress after initial treatment with SU6668. Tumors in some of those animals regrew during the 50 days after cessation of treatment at day 40. After resumption of SU6668 treatment, regression of tumor growth was observed for all of these animals, with rare exceptions (data not shown). These data demonstrate that SU6668 regresses even large tumor xenografts. Furthermore, when sustained regression was not obtained after initial SU6668 treatment, it was achieved in a second round of treatment.

**DISCUSSION**

It has become apparent over the last decade that RTKs are attractive targets for pharmacological intervention. For example, a monoclonal antibody (Herceptin) that targets the RTK HER2 has been approved for the treatment of advanced breast cancer. The elucidation of the three-dimensional structures of kinase domains has led to a greater understanding of the similarities and differences among the various kinase families and has provided insights into the structural features that may be necessary for intervention by small molecules (48). Consequently, numerous small molecule, adenine mimetic inhibitors have been developed that target different RTKs. Several inhibitors are now being further investigated in clinical trials (8, 48, 49), with several more likely to progress to Phase I studies in the near future.

As shown in Table 1, SU6668 has broad activity in biochemical assays. SU6668 is a potent inhibitor of PDGFR kinase activity with a $K_i$ value at least 50× lower than the $K_m$ value of ATP. SU6668 also inhibited Flk-1/KDR and FGFR1 kinase activity. Cell-based assays including HUVEC proliferation and inhibition of tyrosine phosphorylation of these target kinases confirmed the activity of SU6668 against these RTKs (Figs. 3 and 4). However, it is interesting that the low $K_i$ value of SU6668 for PDGFR relative to Flk-1 did not result in a significantly greater ability to inhibit the receptor in cells. It is noteworthy that although the $K_i$ values of SU6668 versus Flk-1 and FGFR are very similar, the inhibition of HUVEC mitogenesis is approximately 20-fold more potent when using VEGF as a ligand than FGF (Fig. 5). Similarly, the inhibition of KDR and Flk-1 phosphorylation in cells is achieved at a lower concentration of SU6668 than inhibition of FRS-2, a substrate phosphorylated by FGFR (Fig. 3). Although we do not fully understand these results, these data illustrate that inhibitory constants derived in the context of purified receptor proteins may not be uniformly translated to receptors replete with additional associated signaling molecules in living cells.

Analysis of the interactions of SU6668 and RTKs by X-ray crystallography and modeling has provided some insight into the differences in SU6668 potency against PDGFR and FGFR. The propionic acid moiety of SU6668 is in a perfect position to interact with the Arg-604 side chain located at the N-lobe of the entrance of the ATP-binding site on PDGFR. In contrast, the corresponding residue of FGFR (Fig. 3). Although we do not fully understand these results, these data illustrate that inhibitory constants derived in the context of purified receptor proteins may not be uniformly translated to receptors replete with additional associated signaling molecules in living cells.

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than the arginine side chain, the interaction between SU6668 on FGFR and Flk-1 would be weaker than that with PDGFR (Fig. 2).

As would be expected of an inhibitor of Flk-1, FGFR1, and PDGFR kinase activity, SU6668 demonstrated significant antitumor activity against a wide range of xenografts (Table 3; Fig. 5). Of particular interest are the tumor types that were poorly inhibited by SU5416, such as the human glioma cell line SF763T (37) and the human ovarian cell line SKOV3TP5. Given its target profile, SU6668 may influence tumor growth by multiple mechanisms including inhibition of endothelial cell proliferation and/or survival as well as tumor cell and stromal cell proliferation. In addition, we cannot preclude the possibility that activity against kinases (as yet unidentified) other than Flk-1/KDR, PDGFR, and FGFR contributes to the biological activity of SU6668.

Strikingly, SU6668 has the ability to induce regression of large established tumors (Fig. 7A). Whereas the mechanism(s) underlying this capability is unknown, the anti-Flk-1/KDR activity of SU6668 is likely to be pertinent, given data implicating VEGF/Flk-1 signaling in the survival of immature blood vessels and cultured endothelial cells (6, 50). Additionally, SU6668 may also impact other host-derived tumor-associated cells such as pericytes and fibroblasts. Pericytes express VEGF and play an indispensable, PDGF-dependent, mechanical role in stabilizing immature blood vessels (51, 52). Fibroblasts may support tumor growth by producing VEGF and are a potential target for PDGF- and FGF-mediated proliferation (53). Consistent with this proposed activity against host-derived cells, SU6668 exhibited potent antiangiogenic activity in glioma xenografts implanted into dorsal skinfold chambers (Fig. 6). In contrast, SU6668 did not potently inhibit the growth of cancer cells in culture (data not shown).

The activity of SU6668 on multiple members of the split RTK family has provided the opportunity to study some key questions concerning inhibitors that target several tyrosine kinases compared...
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with inhibitors that target one kinase specifically, such as SU5416. The attractive and validated targets of SU6668, coupled with its broad, remarkable, activity in tumor xenograft models, have motivated its entry into clinical development. Accordingly, SU6668 has recently entered Phase I clinical trials, and its safety and efficacy profile in humans will emerge in the near future.

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