SU5416 Is a Potent and Selective Inhibitor of the Vascular Endothelial Growth Factor Receptor (Flk-1/KDR) That Inhibits Tyrosine Kinase Catalysis, Tumor Vascularization, and Growth of Multiple Tumor Types

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SU5416, a novel synthetic compound, is a potent and selective inhibitor of the Flk-1/KDR receptor tyrosine kinase that is presently under evaluation in Phase I clinical studies for the treatment of human cancers. SU5416 was shown to inhibit vascular endothelial growth factor-dependent mitogenesis of human endothelial cells without inhibiting the growth of a variety of tumor cells in vitro. In contrast, systemic administration of SU5416 at nontoxic doses in mice resulted in inhibition of subcutaneous tumor growth of cells derived from various tissue origins. The antitumor effect of SU5416 was accompanied by the appearance of pale white tumors that were resected from drug-treated animals, supporting the antiangiogenic property of this agent. These findings support that pharmacological inhibition of the enzymatic activity of the vascular endothelial growth factor receptor represents a novel strategy for limiting the growth of a wide variety of tumor types.

INTRODUCTION

Angiogenesis is the process of sprouting of capillaries from preexisting blood vessels. The overall process is a complex one that involves many biological functions and cell types. Previous research originating from the 1970s has demonstrated that tumor angiogenesis is required for the growth and metastasis of primary solid tumors (1). Endothelial cell activation, migration, and proliferation are major cellular events in this process (2). All of these processes are under the tight regulation of factors that either promote or inhibit angiogenesis. When the balance of these factors is disturbed, unchecked angiogenic factors can be released from hypoxic tumor cells, migrate to the nearby blood vessel endothelia, and signal the activation of the angiogenic response. Tumors that undergo neovascularization can enter a phase of rapid cell growth and may have increased metastatic potential. In the absence of neovascularization, tumors become necrotic (3) and/or apoptotic (4, 5). The importance of angiogenesis in human tumors is reflected by recent studies that demonstrated that the angiogenic phenotype as measured by vessel density is prognostic of survival in people with various types of cancer (6–10).

A number of RTKs are thought to be involved in angiogenesis, either directly or indirectly (11). Of particular interest are the VEGF receptors (Flt-1 and Flk-1/KDR). These receptors are expressed primarily on precursors and mature endothelial cells and have been strongly implicated to play a direct role in angiogenesis associated with human disease (12–14). Germ-line disruption of murine genes for VEGF and its receptors indicated that Flk-1 is required for development of mature endothelial cells (15). Developing embryos derived from Flk-1 --/-- mice lack mature endothelial cells and vessels. In contrast, developing embryos derived from mice containing either the Flt-1 or VEGF homozygous gene disruption exhibited normal endothelial cells, however, with abnormal vasculature (16–18). VEGF and VEGF receptors have been implicated in angiogenesis that occurs in many solid tumors including glioma (19, 20), breast cancer (21), bladder cancer (22), endometrial cancer (23), colon carcinoma (9, 24), and cancers of the gastrointestinal tract (25). A correlation has been observed between VEGF expression and vessel density in human breast tumors (7, 26), renal cell carcinoma (27), and colon cancer (9).

The critical role of Flk-1 in tumor angiogenesis was first demonstrated by using dominant-negative strategies to disrupt the Flk-1 receptor, which resulted in a blockade of endothelial cell mitogenesis as well as inhibition of the growth of eight of nine tumor cell lines implanted subcutaneously into athymic mice (28). In those studies, vessel density was also significantly reduced in the small tumors that did form (28, 29). Other studies using disruption of VEGF expression in embryonic stem cells (18), reduction of VEGF expression using antisense approaches in tumor cells (30, 31), and reduction of VEGF levels using anti-VEGF neutralizing antibodies in tumor cells (32, 33) further defined the critical role of Flk-1 in tumor angiogenesis. Implantation of these embryonic stem cells or tumor cells in mice resulted in inhibition of tumor growth. Mitogenesis of endothelial cells and tumor growth were also inhibited by using neutralizing antibody against Flk-1 (34, 35) and reduction of receptor expression with ribozymes that cleave Flk-1 mRNAs (36).

We sought to develop a synthetic inhibitor of the Flk-1 kinase to block signal transduction through the VEGF receptor. In this regard, we have implemented screening strategies to identify membrane-permeable small synthetic compounds that inhibit the VEGF-dependent phosphorylation of tyrosine residues on the Flk-1 receptor. The following studies describe the identification and characterization of one such compound, SU5416, that has shown good potency and selectivity on the catalytic activity associated with the Flk-1/KDR receptor. In addition, we provide in vivo data that indicate that SU5416 shows a broad antitumor effect, which may be a result of its inhibitory mechanism on tumor angiogenesis.

MATERIALS AND METHODS

Synthesis of SU5416. The chemical name of SU5416 is 3-[2-(4-dimethylpyrrolyl)-5-ylmethylidene]-indolin-2-one, and the structural formula is shown in Fig. 1. The chemical identity of SU5416 is supported by nuclear magnetic resonance, mass spectroscopy, and elemental analysis. SU5416 was prepared from commercially available 3,5-dimethylpyrrolyl-2-carboxaldehyde by aldol-condensation with indolin-2-one in ethanol in the presence of piperidine (37).

Cellular Tyrosine Kinase Assays. NIH 3T3 Flk-1 cells overexpressing Flk-1 receptors (28) were cultured in 10% heat-inactivated FCS in DMEM. NIH 3T3 Flk-1 cells were seeded onto 96-well plates (2 × 10^3 cells/well) in culture medium and cultured overnight, followed by serum depletion in 0.1%
heat-inactivated FCS/DMEM for 24 h. Serial dilutions of SU5416 were added, and the cells were further incubated for 2 h. Tyrosine phosphorylation was stimulated by the addition of 500 ng/ml human recombinant VEGF (Peprotech, Inc., Rocky Hill, NJ) for 5–10 min at 37°C, and the phosphotyrosine content of the immunolocalized receptors was measured as described previously (38) using colorimetric or fluorescence anti-phosphotyrosine antibodies. Cellular kinase assays for PDGF and EGF tyrosine kinase assays were performed as described previously (38). For the measurement of insulin receptor kinase activity, NIH 3T3 cells overexpressing the human insulin receptor (H25) were stimulated with insulin.

**Biochemical Kinase Assays.** Solubilized membranes from 3T3 Flk-1 cells were added to polystyrene ELISA plates that had been precoated with a monoclonal antibody that recognizes Flk-1 (38). After an overnight incubation with lysis at 4°C, serial dilutions of SU5416 were added to the immunolocalized receptor. To induce autophosphorylation of the receptor, various concentrations of ATP were added to the ELISA plate wells containing serially diluted solutions of SU5416. The autophosphorylation was allowed to proceed for 60 min at room temperature and then stopped with EDTA. The amount of phosphotyrosine present on the Flk-1 receptors in the individual wells was determined by incubating the immunolocalized receptor with a biotinylated monoclonal antibody directed against phosphotyrosine. After removal of the unbound anti-phosphotyrosine antibody, avidin-conjugated horseradish peroxidase H was added to the wells. A stabilized form of 3,3',5,5'-tetramethyl benzidine dihydrochloride and H2O2 was added to the wells. The color readout of the assay was allowed to develop for 30 min, and the reaction was stopped with H2SO4. Parallel biochemical kinase assays were performed to measure autophosphorylation on EGFR and fibroblast growth factor receptor.

**Immunoblotting.** 3T3 Flk-1 cells were plated on 24-well dishes and grown to confluency. Dilutions of SU5416 were added and incubated for 1 h at 37°C. Flk-1 autophosphorylation was stimulated by the addition of 100 ng/ml VEGF, and cells were lysed with HNTG [20 mM HEPES (pH 7.5), 150 mM NaCl, 0.2% Triton X-100, and 10% glycerol]. Preparation of lysate, separation of proteins, and immunoblotting with antiphosphotyrosine were performed as described previously (38). For the measurement of insulin receptor kinase activity, NIH 3T3 cells overexpressing the human insulin receptor (H25) were stimulated with insulin.

**Characterization of the Flk-1 Antagonist SU5416**

**Results**

**Identification of SU5416.** In attempts to identify a synthetic molecule that would block the VEGF-dependent kinase activity associated with the Flk-1/KDR receptor, our earlier efforts focused on random screening of small synthetic compounds using a cell-based, VEGF-dependent, Flk-1 tyrosine autophosphorylation assay. Several compound classes that had shown specific inhibitory properties when tested using this Flk-1 autophosphorylation assay had been described previously (38). To focus our attention on those compounds that would be specific antagonists of the Flk-1 kinase, we subjected hundreds of compounds from these and other chemical classes to a series of tests involving whole-cell RTK activity measurements and various ligand-dependent growth assays. As part of this analysis, we identified several indolin-2-ones that showed potent and specific activity in these assays with HUVECs. This provided the rationale to develop a synthesis.
thetetic chemistry effort to diversify and provide additional compounds
to evaluate as potential Flk-1 kinase inhibitors and drug candidates for
the treatment of human cancers. SU5416 was identified during this
process and was found to have many of the features we sought at the
outset.

Selectivity and Potency of SU5416 on Flk-1. SU5416 is a syn-
thetic molecule containing an unsubstituted oxindole core and a
dimethylpyrrole attached to the indolin-2-one at the C3 position (Fig.
1). SU5416 was synthesized and tested in a panel of RTK ELISA-
based assays to determine the relative potency and specificity of this
compound to inhibit tyrosine autophosphorylation on Flk-1. In this
regard, SU5416 was found to inhibit VEGF-dependent phosphoryla-
tion of the Flk-1 receptor in Flk-1-overexpressing NIH 3T3 cells with
an IC$_{50}$ of 1.04 ± 0.53 µM (n = 7). To confirm the inhibitory activity
of SU5416 by immunoblotting, tyrosine phosphorylation associated
with the receptor after ligand stimulation was measured. As shown in
Fig. 2, a dose-dependent decrease in tyrosine phosphorylation of Flk-1
and stimulation of mitogen-activated protein kinase was observed. It
is interesting to note that we observed about a 4-fold more potent
inhibition with SU5416 using the immunoblotting approach compared
with the ELISA assay. This may be due to the measurement of

![Fig. 2](image-url)

**Fig. 2.** NIH 3T3 Flk-1 cells (A) or NIH 3T3 platelet-derived growth factor β cells (B) grown to confluency were preincubated with SU5416 at concentrations ranging from 0.05 to 50 µM for 1 h at 37°C. Receptor phosphorylation was stimulated by the addition of 100 ng/ml VEGF or PDGF-BB, respectively, and cells were lysed with HNTG. Cellular proteins were separated by PAGE and analyzed by immunoblotting using anti-phospho-
tyrosine. MAPK, mitogen-activated protein kinase; PDGFR, PDGFR receptor.

VEGF-driven Mitogenesis of HUVECs. Previous studies have shown that blockade of
VEGF/Flk-1 signaling pathway led to inhibition of endothelial cell
proliferation (28, 34). Therefore, we tested SU5416 for its ability to
inhibit VEGF-driven mitogenesis of HUVECs. As shown in Fig. 4,
SU5416 inhibited VEGF-driven mitogenesis in a dose-dependent
manner with an IC$_{50}$ of 0.04 ± 0.02 µM (n = 3). In contrast, SU5416
blocked FGF-dependent mitogenesis of HUVECs with an IC$_{50}$ of 50
µM (n = 10). This >1000-fold selectivity was consistent with the
inability of SU5416 to block autophosphorylation of isolated FGF
receptors (data not shown). These experiments also substantiated
that SU5416 could block VEGF signaling in human cells and strongly
suggested an inhibition of the tyrosine kinase activity associated with
KDR, the human homologue of the murine Flk-1 receptor tyrosine.

Blockade of the VEGF/Flk-1 pathway had been shown to lead to
inhibition of the subcutaneous growth of tumors (18, 29, 32, 33, 35).
The in vivo efficacy of SU5416 on the growth of subcutaneous tumor
 xenografts was evaluated. A dose-related inhibition of A375 tumor
growth by SU5416 was observed (Fig. 5). In this case, significant
inhibition of subcutaneous tumor growth was observed with daily i.p.
administration of SU5416 in DMSO at 3 mg/kg/day. A >85% inhibi-
tion of tumor growth with no mortality was observed after daily
treatment at 25 mg/kg/day for 38 days. Given this result, we sought to
determine whether there were measurable toxicities at the dose level
of SU5416 that yielded maximal efficacy after chronic treatment in
this model. Blood chemistry parameters reflecting liver (aspartate
aminotransferase and alanine aminotransferase) and kidney (blood
urea nitrogen and creatinine) functions and blood cell counts (WBC
and RBC) from vehicle and drug-treated animals were compared from
several experiments. No significant differences were detected between
the two treatment groups (data not shown). Treatment of animals at 25
mg/kg/day resulted in a transient body weight loss after the first 7
days of dosing and was regained by 2–3 weeks after initiation of drug
treatment. Histopathological analysis of the major organs (heart, lung,
kidneys, liver, and intestine) from animals treated with SU5416 at 25

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**Characterization of the Flk-1 Antagonist SU5416**

SU5416 is a synthetic molecule containing an unsubstituted oxindole core and a dimethylpyrrole attached to the indolin-2-one at the C3 position (Fig. 1). SU5416 was synthesized and tested in a panel of RTK ELISA-based assays to determine the relative potency and specificity of this compound to inhibit tyrosine autophosphorylation on Flk-1. In this regard, SU5416 was found to inhibit VEGF-dependent phosphorylation of the Flk-1 receptor in Flk-1-overexpressing NIH 3T3 cells with an IC$_{50}$ of 1.04 ± 0.53 µM (n = 7). To confirm the inhibitory activity of SU5416 by immunoblotting, tyrosine phosphorylation associated with the receptor after ligand stimulation was measured. As shown in Fig. 2, a dose-dependent decrease in tyrosine phosphorylation of Flk-1 and stimulation of mitogen-activated protein kinase was observed. It is interesting to note that we observed about a 4-fold more potent inhibition with SU5416 using the immunoblotting approach compared with the ELISA assay. This may be due to the measurement of tyrosine phosphorylation in the Flk-1 immune complex that is not due to Flk-1 autophosphorylation. The immunoblotting experiments measured more precisely only phosphorylation on Flk-1, because the receptor complex was resolved from other cellular proteins by electrophoresis before the detection of tyrosine phosphorylation.

The selective activity of SU5416 on Flk-1 was supported by the fact that testing of SU5416 using NIH 3T3 cells overexpressing either the EGF or insulin receptors indicated a complete lack of activity (IC$_{50}$ > 100 µM). This observation was confirmed by immunoblotting after ligand stimulation (Fig. 3). An IC$_{50}$ of 20.25 ± 5.2 µM (n = 7), which is about 20-fold less in potency on PDGF-dependent autophosphorylation, was observed when SU5416 was tested in NIH 3T3 cells overexpressing the human PDGF receptor β. As in the case of Flk-1, we also observed a more potent activity of SU5416 to inhibit PDGF-dependent phosphorylation by immunoblotting compared with the ELISA data (Fig. 2). To ascertain whether SU5416 directly affected the catalytic function of the Flk-1 receptor, the effect of SU5416 on autophosphorylation of the isolated receptor was examined. SU5416 was shown to inhibit autophosphorylation of the Flk-1 receptor with an IC$_{50}$ of 1.23 ± 0.2 µM (n = 4) using an ELISA-based biochemical kinase assay. Comparable IC$_{50}$ values of SU5416 on the Flk-1 cellular and biochemical kinase assays suggested that the inhibition observed on phosphorylation was probably not due to indirect effects such as interference with binding of ligand but rather a direct effect on the kinase. Testing of SU5416 using isolated EGF and FGF receptor tyrosine kinases revealed no inhibitory activity using similar biochemical assays (IC$_{50}$ > 100 µM). The lack of inhibitory activity with FGF receptor was confirmed by immunoblotting (Fig. 3).

Selectivity and Potency of SU5416 on VEGF-driven Mitogenesis of HUVECs. Previous studies have shown that blockade of VEGF/Flk-1 signaling pathway led to inhibition of endothelial cell proliferation (28, 34). Therefore, we tested SU5416 for its ability to inhibit VEGF-driven mitogenesis of HUVECs. As shown in Fig. 4, SU5416 inhibited VEGF-driven mitogenesis in a dose-dependent manner with an IC$_{50}$ of 0.04 ± 0.02 µM (n = 3). In contrast, SU5416 blocked FGF-dependent mitogenesis of HUVECs with an IC$_{50}$ of 50 µM (n = 10). This >1000-fold selectivity was consistent with the inability of SU5416 to block autophosphorylation of isolated FGF receptors (data not shown). These experiments also substantiated that SU5416 could block VEGF signaling in human cells and strongly suggested an inhibition of the tyrosine kinase activity associated with KDR, the human homologue of the murine Flk-1 receptor tyrosine.

Blockade of the VEGF/Flk-1 pathway had been shown to lead to inhibition of the subcutaneous growth of tumors (18, 29, 32, 33, 35). The in vivo efficacy of SU5416 on the growth of subcutaneous tumor xenografts was evaluated. A dose-related inhibition of A375 tumor growth by SU5416 was observed (Fig. 5). In this case, significant inhibition of subcutaneous tumor growth was observed with daily i.p. administration of SU5416 in DMSO at 3 mg/kg/day. A >85% inhibition of tumor growth with no mortality was observed after daily treatment at 25 mg/kg/day for 38 days. Given this result, we sought to determine whether there were measurable toxicities at the dose level of SU5416 that yielded maximal efficacy after chronic treatment in this model. Blood chemistry parameters reflecting liver (aspartate aminotransferase and alanine aminotransferase) and kidney (blood urea nitrogen and creatinine) functions and blood cell counts (WBC and RBC) from vehicle and drug-treated animals were compared from several experiments. No significant differences were detected between the two treatment groups (data not shown). Treatment of animals at 25 mg/kg/day resulted in a transient body weight loss after the first 7 days of dosing and was regained by 2–3 weeks after initiation of drug treatment. Histopathological analysis of the major organs (heart, lung, kidneys, liver, and intestine) from animals treated with SU5416 at 25
mg/kg/day indicated no significant lesions in any of these major organs (data not shown). We concluded that SU5416 treatment of animals resulted in good efficacy without measurable toxicity.

Broad Spectrum Antitumor Activity of SU5416. The growth of a variety of tumors including glioma, lung, and ovarian carcinomas had been shown to be inhibited after the blockade of the VEGF/Flk-1 pathway with a dominant-negative form of the Flk-1 receptor (29). Therefore, one might predict that a small molecule inhibitor of Flk-1 would block the growth of a wide variety of tumor types. Consequently, the activity of SU5416 was measured against the growth of various tumor types (Table 1). SU5416 significantly inhibited the subcutaneous growth of 8 of 10 tumor lines tested with an average mortality rate of 2.5%. The broad tumor efficacy of SU5416 was consistent with that observed after the obliteration of the Flk-1 signaling pathway by the use of Flk-1 dominant-negative mutants (29).

Antitumor Activity of SU5416 Correlates with an Antiangiogenesis Mechanism. To rule out the possibility that SU5416 had an effect upon the growth of tumor cells directly, the in vitro growth inhibitory properties of SU5416 on selected tumor cell lines used in the in vivo studies was tested. SU5416 treatment had no effect on the in vitro growth of C6 glioma, Calu 6 lung carcinoma, A375 melanoma, A431 epidermoid carcinoma, and SF767T glioma cells (all IC50s > 20 μM). Given the 500-fold more potent growth-inhibitory properties of SU5416 on endothelial cells, we concluded that the inhibitory effect of SU5416 may be mediated by a direct effect on the the angiogenic process associated with tumor growth.

To assess whether the efficacy of SU5416 on inhibition of local tumor growth would vary with implant sites and vascular beds, tumor cells were implanted under the serosal layer of the colon, and the efficacy of SU5416 was evaluated. A series of tumor cell lines derived from various tissue origins including WiDR, Colo320, PancTu, Eph4...
**DISCUSSION**

These present studies support SU5416 as a potent and specific inhibitor of the Flk-1 tyrosine kinase. It is important to note that a related molecule, SU5402, has been synthesized and co-crystallized within the catalytic core of the FGF receptor-1 (fgf1; Ref. 42). Elucidation of the crystallographic structure of this 3-substituted indolin-2-one core occupies a site in which the adenine of ATP binds, and the substituent at the C-3 position of the indolin-2-one core was shown to extend into the hinge region between the two kinase lobes. We have performed biochemical experiments with SU5416 that indicated that its inhibitory properties on Flk-1 may be ATP dependent (data not shown), suggesting ATP mimetic properties as well. The crystallographic studies are consistent with studies using 4-anilinoquinazolines, for which it has been shown that highly potent and specific inhibitors of the EGF RTK inhibits the kinase in an ATP-competitive manner by localizing in the ATP binding pocket of the EGFR TK (43). Biochemical characterization of SU5402 has revealed that it specifically inhibits the function of the FGF receptor when compared with inhibition of kinase activity of the PDGF, EGF, and insulin receptors. The specificity of this molecule has been suggested to result from a strong hydrophobic interaction of the pyrrole ring system in conjunction with an electrostatic interaction of the carboxyethyl moiety of the compound with Asn 568 in the hinge region of the nucleotide binding domain of the kinase. A less specific indolin-2-one, SU4984, was shown not to exhibit the electrostatic interaction and did not contain a pyrrole at the C-3 position of the indolin-2-one. Unlike SU5402, SU5416 did not show inhibitory activity on autophosphorylation on the FGF RTK or against FGF-driven proliferation in human endothelial cells. It was surprising that replacement of the propionic acid substituent by a methyl group and the movement of the second methyl group from the c-4’ position to the c-5’ position on the pyrrole would have resulted in such a pronounced specificity for the VEGF receptor, given that this would not favor electrostatic interactions that would be specific to VEGF receptors. In addition to the VEGF receptors, we have also shown in this report that SU5416 exhibits inhibitory properties against the PDGF receptor. Because PDGF has been implicated to play a role in angiogenesis either via induction of VEGF (44, 45) or as a direct growth enhancer on pericytes (46) and fibroblasts (47, 48) surrounding the endothelial cells, inhibition of PDGF RTK activity by SU5416 may contribute to its antiangiogenic properties.

Although it is clear that our focus at the outset was to block the function of Flk-1/KDR on endothelial cells, it is also possible that other VEGF receptors, such as Flt-1 and Flt-4, may be affected by SU5416. In this regard, it has been shown that SU5416 blocks VEGF-induced Flt-1 activity.\(^4\) This aspect might be expected given the substantial amino acid homology in the ATP binding pocket when the VEGF receptors are compared. Although reduction of endothelial mitogenesis after the blockade of the VEGF/Flt-1 pathway (36, 49) has been reported, the direct role of Flt-1 signaling in mitogenesis remains unclear because mutant forms of VEGF that had reduced binding to Flt-1 but normal binding to Flk-1 stimulated endothelial

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\(^4\) M. Clauss and W. Risau, unpublished data.
SU5416 treated

vehicle control

Fig. 6. Rat C6 glioma cells were surgically implanted (0.5 × 10^6 cells/animal) under the serosa of the colon in BALB/c nu/nu mice. Beginning 1 day after implantation, animals were treated once daily with a 30 μl i.p. bolus injection of either SU5416 at 25 mg/kg/day in DMSO or DMSO alone for 16 days. On day 16 after implantation, animals were euthanized, and their local tumors in the colon were first quantitated by measurement using venier calipers and then harvested. A 73% decrease in tumor volume was recorded in the drug-treated group (P < 0.00001) and was calculated by comparing mean tumor size of the treated groups versus mean tumor size of the vehicle control group using Student’s t test. Representative tumors from SU5416-treated (top) and DMSO-treated (bottom) animals are shown.

cells similar in animals to wild-type VEGF (50). Results from gene disruption studies also suggested that Flt-1 may be involved in the interaction of endothelial cells and the matrix required for normal vessel assembly (16). In addition to mediating a mitogenic signal on endothelial cells, activation of Flt-1 on these cells resulted in the production of tissue factor, a protein primarily responsible for initiation of blood coagulation (51). Tissue factor is also a monocyte chemoattractant and is produced by monocytes after stimulation of Flt-1 (51). Therefore, inhibition of Flt-1 signaling by SU5416 may lead not only to interference with the formation of the endothelial-matrix interactions but also monocytic-dependent inflammatory responses and attenuation of the thrombosis often associated with cancer malignancies.

During the course of conducting these studies, we found that the efficacy of SU5416 after daily i.p. dosing was dependent on the growth rate of the tumors and was more optimal against slower growing tumors (<1000 mm^3 over a 30-day period after implantation) and more variable against fast-growing tumors (>1000 mm^3 within 14-days after implantation). This effect may be due to differences in the requirement of new blood vessels for the growth of particular tumors and the angiogenic factors produced by a particular tumor cell population. With regard to this angiogenesis requirement, the ability to inactivate the Flk-1 receptor may be a consequence of receptor turnover and the availability of SU5416 to inactivate the receptor prior to cells entering the S phase of the cell cycle. Although a single exposure of SU5416 has a duration of action of >24 h on the VEGF-dependent inhibition of proliferation of HUVECs, it is unclear how long the VEGF receptor may remain inactivated once SU5416 is bound in the active site. Given the fact that a once-a-day dosing regimen is efficacious, although detection of SU5416 in the blood is short-lived (data not shown), it is suggestive that the biological half-life of SU5416 might be long. In cases where the angiogenic process and VEGF receptor turnover exceeds the pharmacological inactivation of the receptor, we would predict less efficacy with the daily regimen. This aspect may help to explain our observation that SU5416 was found to be ineffective against the subcutaneous growth of SF763T and SF767T tumors. Alternatively, the reduced efficacy of SU5416 on the growth of these tumors may be a reflection of the use of angiogenic factors other than VEGF that may be operative in the growth of these tumors. These tumor-specific differences may not necessarily reflect differences in the factors produced by a given tumor but rather a switching of VEGF to non-VEGF angiogenic factors after the tumors have reached a certain size. Recent studies, using a tetracycline-regulated system in which expression of VEGF can be effected, have shown that the requirement of VEGF for in vivo growth of a human breast carcinoma cell line was dependent on the size of the tumors. It was proposed that when these tumors have reached a certain size, VEGF may not be essential for supporting tumor growth and other angiogenic factors, such as bFGF and transforming growth factor α, may substitute for VEGF (52). Therefore, the development of inhibitors with activity against FGF or other receptors may be warranted for the treatment of those tumor types that may be predisposed to such an effect.

Angiogenesis is defined as the sprouting of new vessels from existing vasculature and encompasses a complex process involving many biological functions. It is generally characterized by vasodilatation, increased protein leakage, remodelling of the extracellular matrix, interaction of endothelial cells with newly synthesized integrins, up-regulation of growth factor receptors, differentiation and shape changes of endothelial cells, and recruitment of pericytes and smooth muscle cells, followed by the deposition of new matrix proteins for tube formation (53). During development, the angiogenic process is active to ensure the formation of a network of capillaries required for embryonic growth but essentially ceases during adult life. The turnover of endothelial cells in the normal human adult is very low, in the order of years, except during corpus luteum formation, pregnancy, wound healing, or when oxygen supply is compromised. Therapeutic strategies aimed at inhibiting various steps in the process of angiogenesis are under preclinical and clinical evaluation (54). Most of these agents interfere with the response of endothelial cells to angiogenic peptides; some inhibit the activity of matrix-metalloproteinases related to the increased invasive, metastatic, and angiogenic potential of tumors, and other agents directly target or destroy the

5 R. Schreck and T. A. T. Fong, unpublished data.
vasculature. In patients, these approaches may result in small avascular tumors maintained in a dormant state, and such therapies may have increased safety features compared with conventional cytotoxic therapy. Use of an inhibitor of VEGF receptors such as SU5416 would be distinct from the mechanisms of these anti-angiogenesis agents mentioned above and may be complementary to these agents because the mechanistic rationale for SU5416 treatment is distinct.

An inhibitor of the VEGF receptor would be predicted to have a significant therapeutic benefit to patients without the substantial side effects associated with conventional cytotoxic therapy. SU5416 represents the first synthetic inhibitor of VEGF receptor function to enter clinical studies and represents an opportunity to test mechanism-based, anti-angiogenic therapy. Clearly, it has the potential for treatment of cancers and their metastases. In addition, development of small molecule inhibitors of the VEGF receptor function may also have the potential to affect VEGF-mediated processes associated with a wide variety of diseases associated with pathological angiogenesis such as diabetic retinopathies, psoriasis, rheumatoid arthritis, and endometriosis. SU5416 and related compounds may be useful agents for the treatment of these diseases as well.

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