Flk-1 as a Target for Tumor Growth Inhibition


ABSTRACT

A number of growth factor receptor tyrosine kinases have been implicated in angiogenesis, including epidermal growth factor receptor, fibroblast growth factor receptor, platelet-derived growth factor receptor, Flk-1/KDR, Flt-1, Tie-1, and Tek/Tie-2. Flk-1/KDR, a receptor for vascular endothelial growth factor (VEGF), is expressed exclusively in endothelial cells. Using dominant-negative methods, Flk-1 was shown to play a role in angiogenesis and the growth of a variety of tumor types. Because of this, a drug discovery effort was established to identify Flk-1 kinase inhibitors. For initial screening, an ELISA in a 96-well format was used to measure VEGF-induced Flk-1 tyrosine phosphorylation in whole cells. Compounds that inhibited ligand-induced receptor autophosphorylation were confirmed by antiphosphotyrosine immunoblotting. Inhibition of VEGF-stimulated DNA synthesis in human endothelial cells was also assessed. Inhibitors were further evaluated for their effects on vessel formation using the chorioallantoic membrane assay. Using these methods, antiangiogenesis compounds that inhibit Flk-1 tyrosine kinase activity, endothelial cell mitogenesis, and blood vessel formation in the chorioallantoic membrane assay have been found.

INTRODUCTION

Angiogenesis is a multistep process that results in the formation of new blood vessels from preexisting vasculature (for review, see Ref. 1). During angiogenesis, endothelial cells release enzymes that degrade the basement membrane, migrate through the membrane to form a sprout, and proliferate to extend the vessel. This process is required for the growth of tumors beyond a minimum volume. Many growth factors and their receptors act as stimulators or inhibitors for the various steps in this complex process. Fibroblast growth factor and EGF receptors transmit signals for chemotaxis and mitogenesis of endothelial cells (2). Flk-1/KDR and Flt-1, the receptors for VEGF, were shown by in situ hybridization to be expressed exclusively on endothelial cells (3–5). Flk-1 is involved in VEGF-induced mitogenesis, but the role of Flt-1 is not clear (6). A new family of tyrosine kinase receptors consisting of Tie-1 and Tek/Tie-2 has also been found on endothelial cells. Although their ligands and exact roles have not been identified, the patterns of expression (7, 8) and the results of targeted mutagenesis in mice (9) suggest that they are involved in the angiogenic process.

The restricted expression of Flk-1 and its human homologue KDR to the endothelium suggests that it plays an important role in angiogenesis (3, 4, 10). Previously, in vivo dominant-negative experiments were used to demonstrate that specific inhibition of Flk-1 suppressed s.c. and intracerebral growth of a variety of solid tumor types in rodents (11, 12). The mechanism appeared to be by inhibition of angiogenesis, indicating that VEGF/Flk-1 is the dominant pathway for angiogenesis in solid tumors. This is supported by other studies that showed inhibition of tumor cell growth in athymic mice by anti-VEGF monoclonal antibodies (13) or anti-VEGF antisense RNA expression (14).

Recently, VEGF was shown to play a role in diabetic retinopathy (15), psoriasis (16), and hemangioblastoma (17), as well as cancer. Thus, identification of Flk-1/KDR inhibitors may be useful for treatment of a variety of disease states in which angiogenesis is involved. A drug discovery program was established to identify small molecule compounds that specifically inhibit the enzymatic activity of Flk-1 kinase and downstream events.

MATERIALS AND METHODS

Synthesis of Compounds

All compounds were characterized by standard analytical methods (nuclear magnetic resonance and mass spectrometry).

SU8079 [(E)-3-(3, 5-di-tert-Butyl-4-hydroxyphenyl)-2-aminothiocar- bonyl]acrylonitrile. This was synthesized as described previously (18).

SU1433 [2-(3,4-Dihydroxyphenyl)-6,7-dimethylquinazoline-HCl]. 4,5-Dimethyl-1,2-phenylene diamine (1.9 g) and α-chloro-3,4-dihydroxyacetophenone (1.9 g) were heated to reflux in 25 ml ethanol for 2 h. Cooling and filtering yielded the product (melting point, 278°C).

SU1498 [(E)-3-(3,5-Diisopropyl-4-hydroxyphenyl)-2-(3-phenyl-n-propylaminocarbonyl]acrylonitrile. 3,5-Diisopropyl-4-hydroxy-benzalde- hyde(4.12 g) and N-(3-phenyl-n-propyl)cyanoacetamide (4.24 g) were heated to reflux for 5 h in ethanol (40 ml) containing 0.5 ml piperidine. The mixture was poured into 200 ml diluted HCl solution and extracted with methylene chloride (2 × 150 ml). Workup yielded a crude solid, which was recrystallized from toluene to provide the product.

SU1835 [2-Benzyl-1-(4-hydroxyphenyl)-3-methyl-2,3-dihydro-1'H-im- idazo[5,1-b]quinazolin-9-one]. Bromine (1.6 g) was added dropwise to 2-ethylquinazolin-4-one (1.62 g) and sodium acetate (0.82 g) in acetic acid (20 ml) at room temperature and stirred 2 h. The resulting 2-(1-bromoethyl)quinazolin-4-one (1.95 g) was recovered by filtration, and mixed with 15 ml water and benzylamine (2.2 ml). The mixture was heated in a water bath until the solid disappeared, neutralized with acetic acid, and put in the refrigerator to crystallize. The 2-(1-benzylamino-ethyl)quinazolin-4-one (2.0 g) was filtered out, dried and mixed with 4-hydroxybenzaldehyde (0.88 g). These were fused under nitrogen at 160°C for 15–30 min until the H2O formed had distilled out. The solidified mixture was recrystallized from ethanol three times to provide the product (melting point, 226–228°C).

SU1436 [(E)-2-Cyanomethylsulfonfyl-3-(3,5-diisopropyl-4-hydroxyph- enyl)acrylonitrile]. A mixture of 3,5-diisopropyl-4-hydroxy-benzaldehyde (500 mg) and sulfonfyl diacetoneitrile (700 mg) in 6 ml ethanol and a few drops of piperidine was heated to reflux with for 4 h. Workup resulted in a crude product, a portion of which was purified by high-performance liquid chromatography on a C-18 column to provide SU4136.

Cells and Cell Culture

All reagents and media for cell culture were obtained from Life Technolo- gies, Inc. (Gaithersburg, MD). 3T3 Flk-1 cells were engineered by infecting NIH 3T3 cells with virus carrying wild-type flk-1 (11). They were cultured in 10% FBS in DMEM. HUV-ECs were purchased from the American Type...
Culture Collection (Rockville, MD) and cultured in 10% heat-inactivated FBS/F-12K nutrient mixture supplemented with 5 ng/ml endothelial cell growth factor (Boehringer Mannheim, Indianapolis, IN).

Cellular Kinase Assay

3T3 Flk-1 cells were plated onto 96-well plates (2.5 × 10⁴ cells/well) in 10% FBS/DMEM and cultured overnight, followed by serum depletion in 0.1% FBS/DMEM for 24 h. Serial dilutions of compounds were added, and the cells were incubated for 1 h. Phosphorylation was stimulated by the addition of 50 ng/ml human recombinant VEGF (Peprotech, Inc., Rocky Hill, NJ) and 1 mM Na₃VO₄. After 8 min at 37°C, cells were washed with PBS and lysed with HNTG (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.2% Triton X-100, and 10% glycerol) plus 5 mM Na₂VO₄, 2 mM Na₃PO₄, and 5 mM EDTA. Lysates were transferred to 96-well plates precoated with polyclonal anti-Flk-1 antibody (anti-Flk-1D) and incubated 2 h. Phosphoryrosine was detected by a monoclonal anti-phosphotyrosine antibody and peroxidase-conjugated anti-mouse IgG. Substrate was H₂O₂ with 0.5 mg/ml 2,2'-azino-bis(3-ethylbenz-thiazoline)-sulfonic acid (Sigma Chemical Co., St. Louis, MO) in 100 mM citric acid and 250 mM Na₂HPO₄ (pH 4).

ELISAs for other tyrosine kinase receptors were carried out by similar procedures. PDGF and EGFR receptors were expressed in NIH 3T3 cells and stimulated with the corresponding ligands. For measurement of HER-2 kinase activity, a chimera made of the extracellular domain of the EGF receptor and transmembrane and intracellular domains of HER-2 was expressed on NIH 3T3 cells and stimulated with EGF. After treatment of the cells with compounds and ligands, the receptors were bound to ELISA plates by antibodies against the extracellular domains, and phosphoryrosine was detected as described for the Flk-1 assay.

Immunoblotting

3T3 Flk-1 cells were plated on 10-cm dishes, grown to confluence, and serum depleted overnight in 0.1% FBS/DMEM. Dilutions of compounds were added in 5 ml medium and incubated for 2 h at 37°C. Phosphorylation was stimulated by the addition of 50 ng/ml VEGF, and cells were lysed with 1 ml HNTG/plate. Flk-1 was immunoprecipitated with 4 μg/ml anti-Flk-1D and protein A-agarose. Reduced samples were run on 7.5% polyacrylamide gels, transferred to nitrocellulose, and blotted with antiphosphotyrosine. The presence of Flk-1 was confirmed by stripping the blots and reblotting with anti-Flk-1D. Detection was with peroxidase-conjugated anti-IgG and enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL).

Endothelial Cell Mitogenesis Assay

Endothelial cells (HUVEC) were plated onto 96-well plates (10⁴ cells/well) in 0.5% heat-inactivated FBS/F-12K and incubated 24 h. Serial dilutions of compounds were added with 20 ng/ml VEGF in 0.5% FBS. After 24 h, [³H]thymidine (1 μCi/well; Amersham) was added, and the cells were incubated for another 24 h and frozen at −20°C overnight. Cells were thawed and harvested onto filter mats with a Tomtec Harvester 96 and counted with a Wallac 1205 Betaplate liquid scintillation counter.

Chorioallantoic Membrane Assay

Fertilized eggs (California Golden Eggs, Sacramento, CA) were opened at day 3, poured into sterile glass dishes, and returned to a 37°C humidified incubator. Compounds were prepared by resuspending in water or methanol and mixing 1:1 with 1% methylcellulose (Sigma) in sterile water. Pellets containing 10–100 μg compound in 0.5% methylcellulose were made by pipetting 10 μl of the compound mixtures onto plastic Petri dishes and drying at ambient temperature. The pellets were placed with forceps on the CAM at days 4–6 of development, and the effects were observed through day 10 or 11. At the end of the experiments, pellets were soaked with PBS and removed from the membranes with forceps. The CAMs were fixed with Bouin’s solution (Newcomer Supply, Middleton, WI), cut out, and dried onto polylsine-coated slides. The Bouin’s solution was removed with 70% ethanol, and the membranes were treated with diaminobenzidine (Sigma) for 5 min to stain for endogenous peroxidase in RBC.

Vessel Permeability Assay

All procedures with animals followed the NIH Guide for the Care and Use of Laboratory Animals, and the facility has been accredited by the American Association for the Accreditation of Laboratory Animal Care.

VEGF was prepared as time-release pellets by Innovative Research of America (Toledo, OH), providing approximately 10 or 100 ng/day VEGF. Methycellulose pellets were made as for the CAM assay, except that compound suspensions were mixed 1:9 with 1.5% methylcellulose, and 25-μl aliquots were dropped onto parafilm and dried. The resulting pellets contained 50 μg compound in 1.35% methylcellulose. BALB/c-nu/nu (athymic) mice (6–10 weeks; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were anesthetized with isofluorane. A 10-gauge trochar was used to place a VEGF or placebo pellet s.c. on the ventral side near the xyphoid process. A methylcellulose pellet containing compound (50 μg/animal) or carrier only was placed about 1.5 cm lateral to the VEGF pellet. Animals (four to eight mice per group) were observed approximately 24 h later when pellet implant sites were evaluated for redness and local response.

RESULTS

A high-throughput, 96-well format ELISA was established to screen for inhibitors of Flk-1 kinase activity. NIH 3T3 cells expressing Flk-1 (3T3 Flk-1 cells) were stimulated with VEGF, and lysates of the cells were subjected to a sandwich ELISA with antibodies against Flk-1 and phosphotyrosine. A 2–4-fold increase in signal was detected compared with untreated cells. Incubation of the cells with dilutions of compounds led to the discovery of a number of potent kinase inhibitors (Fig. 1). These possessed IC₅₀ values varying from 0.7 to 9.3 μM for inhibition of tyrosine phosphorylation on Flk-1 (Table 1). To evaluate their specificity for Flk-1, the compounds were also assayed in similar ELISAs that measured PDGF receptor, EGFr receptor, and HER-2 kinase activity. These receptors were also expressed in NIH 3T3 cells; therefore, all assays were performed in the same cellular background. SU1433 had an IC₅₀ value of 5 μM in the PDGF receptor assay, but none of the other compounds inhibited significantly in the concentration ranges tested (Table 1).

The phosphorylation state of Flk-1 was also examined using im-

![Fig. 1. Structures of Flk-1 kinase inhibitors.](cancerres.aacrjournals.org)
munoblotting. This was to confirm that the decreased signal in the ELISA was due to inhibitory effects of the compounds on tyrosine phosphorylation of Flk-1 and not from decreased phosphorylation of associated proteins or to loss of Flk-1 protein. Cells were treated as described for the ELISA, and Flk-1 was immunoprecipitated before blotting. As shown in Fig. 2A, treatment of 3T3 Flk-1 cells with 50 ng/ml VEGF increased the tyrosine phosphorylation on Flk-1 5-10-fold. All five compounds decreased the phosphotyrosine signal at 10 and 50 μM, and SU0879 reduced the signal at 5 μM as well. No other bands were visible. The blots were stripped and rebotted with anti-Flk-1 antibody to verify that the loss in phosphotyrosine was not due to a loss of Flk-1 protein (Fig. 2B). The compounds identified by the ELISA inhibited phosphorylation of Flk-1 and not phosphorylation of associated proteins.

Inhibitors of Flk-1 kinase activity were further evaluated in mitogenesis assays using human endothelial cells. HUV-ECs were used because they naturally express KDR, the human homologue of Flk-1, and respond to VEGF (19, 20). Preliminary experiments established that endothelial cells incubated with 20 ng/ml VEGF followed by [3H]thymidine routinely incorporated 5-10-fold more thymidine than cells without VEGF. All the Flk-1 kinase inhibitors identified by the ELISA blocked thymidine incorporation in response to VEGF, with IC50 values ranging from 0.6 to 10 μM (Table 1). This confirmed that inhibitors of Flk-1 kinase activity also inhibited a relevant downstream response to VEGF in human cells.

CAM assays were used to determine the effects of the Flk-1 inhibitors on angiogenesis. The compounds were prepared in methylcellulose pellets and applied to the CAMs of 4-6-day-old chicken embryos. SU1433 and SU1498 prevented the formation of new vessels under the pellets (Fig. 3). Membranes exposed to pellets made with either methanol or water alone and areas away from the compound pellets were not affected. SU1835 had no effect, and SU0879 and SU4136 caused scarring of the membrane. Some inhibitors of Flk-1 kinase activity also prevented angiogenesis in the CAM.

VEGF serves as a vascular permeability factor as well as a growth factor (21). To assay for this function, VEGF was prepared in time-release pellets and implanted s.c. on the ventral sides of athymic mice. Within 1 day, the abdomens of the mice became red and swollen; the effect was greater with the higher dose (Fig. 4, B and C). The mice implanted with placebo pellets were not affected (Fig. 4A).

Compounds were tested by preparing them in methylcellulose pellets and implanting them near VEGF-releasing pellets. SU1433 inhibited the response seen in animals implanted with VEGF (Fig. 5B). SU1498 also inhibited the reddening response (Fig. 5D). The compounds alone did not have any visible effects (Fig. 5, C and E). Therefore, these inhibitors appeared to prevent vascular permeability as well as angiogenesis, both of which are known to be mediated by VEGF.

### Table 1

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<th>Compound</th>
<th>Flk-1 kinase</th>
<th>PDGF-receptor kinase</th>
<th>EGF-receptor kinase</th>
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**DISCUSSION**

Inhibitors of angiogenesis have recently been identified in a variety of systems. Reintroduction of wild-type p53 into glioblastoma cells caused the release of an inhibitor of vessel formation (22). In another model, angiostatin was identified as a suppressor of metastasis and was found to inhibit angiogenesis (23). Its mode of action appears to be through inhibition of fibroblast growth factor-stimulated growth of endothelial cells. In small metastatic tumors, the rate of cell proliferation appears to be balanced with apoptosis; the balance is shifted only when the tumor becomes angiogenic (24). This opens up the possibility of treating patients with antiangiogenesis agents to prevent the growth of tumors and metastases.
Flk-1 AS A TARGET FOR TUMOR GROWTH INHIBITION

Angiogenesis and growth and thus may be good targets for therapeutic intervention. VEGF is expressed by a variety of tumor cell lines in culture (20, 30, 31). It has also been shown by in situ hybridization to be present in human glioblastomas, especially near hypoxic regions (32, 33). VEGF appears to be involved in the growth of tumors; rat glioma cells engineered to express antisense VEGF RNA no longer formed s.c. tumors in athymic mice (14). Additionally, a neutralizing monoclonal antibody against VEGF inhibited the growth of human tumor cells implanted into mice (13). Inhibition of the VEGF receptor

Potentially, inhibition of any of the steps of angiogenesis will prevent tumor growth. Progress has recently been made in this area (for review, see Ref. 25). Linomide blocked prostatic cancer growth in rats, presumably by acting as an antichemotactant and cytostatic factor for endothelial cells (26). Inhibition of the growth of endothelial cells (27) by the fumagillin derivative AGM-1470 prevented tumor growth and metastasis (28). The teratogen thalidomide was found to inhibit angiogenesis and is under investigation as an antitumor agent (29).

VEGF and Flk-1 have been implicated by several methods in tumor angiogenesis and growth and thus may be good targets for therapeutic intervention. VEGF is expressed by a variety of tumor cell lines in culture (20, 30, 31). It has also been shown by in situ hybridization to be present in human glioblastomas, especially near hypoxic regions (32, 33). VEGF appears to be involved in the growth of tumors; rat glioma cells engineered to express antisense VEGF RNA no longer formed s.c. tumors in athymic mice (14). Additionally, a neutralizing monoclonal antibody against VEGF inhibited the growth of human tumor cells implanted into mice (13). Inhibition of the VEGF receptor
Flk-1 also significantly reduced the growth of a variety of tumor cell types in \textit{in vivo} dominant-negative experiments (11, 12).

Flk-1 is autophosphorylated within minutes after treatment with VEGF, and this phosphorylation is required for downstream signaling (4, 34). Thus, compounds that reduce tyrosine phosphorylation on Flk-1 should block its activity. An ELISA that measures phosphotyrosine on Flk-1 was developed to screen compound libraries. Inhibitory compounds were also assayed for their effects on the kinase activities of the PDGF receptor, EGF receptor, and HER-2. All of the receptors were expressed in NIH 3T3 cells; therefore, the cellular background was the same for all assays. Screening by ELISAs resulted in the identification of many compounds that specifically inhibited Flk-1 phosphorylation. The PDGF receptor, which is more closely related to Flk-1 than the other receptors assayed, was inhibited by SU1433, but the other compounds were very specific for Flk-1. Compounds with IC$_{50}$ values lower than 20 $\mu$m in the Flk-1 kinase assay (Fig. 1) were further analyzed by antiphosphotyrosine Western blotting. The only strong protein band seen on blotting immunoprecipitations from VEGF-treated cells was of the expected size for Flk-1. Inhibitors found in the ELISA caused a decrease in the intensity of this band, which was not due to a loss of Flk-1 protein, as shown on the blots reprobed with an anti-Flk-1 antibody. Thus, the inhibitory effects of the compounds are likely to be a result of Flk-1 tyrosine kinase inhibition.

It has been shown that VEGF binds to Flt-1 as well as Flk-1 (35). The exact roles of Flk-1 and Flt-1 are not clear, but there is some evidence that they do not serve the same function. Porcine aortic endothelial cells engineered to express KDR underwent actin reorganization, chemotaxis, and mitogenesis in response to VEGF, whereas cells expressing Flt-1 did not (6). There are also conflicting reports about the kinase activity of Flt-1 (6, 35, 36). Furthermore, targeted mutagenesis of \textsc{flk-1} in mice prevented the development of mature endothelial cells (37). A similar mutation in \textsc{flt-1} did not affect endothelial cells, but the formation of normal vessels was impaired (38). To eliminate the possibility of identifying inhibitors of Flt-1 that do not also inhibit Flk-1, NIH 3T3 cells engineered to express Flk-1 (3T3 Flk-1 cells) were used to identify and evaluate compounds.

Inhibitors of Flk-1 were further analyzed for their effects on VEGF-mediated downstream events. Endothelial cells (HUV-EC) were chosen for subsequent studies because they are the cellular targets for intervention and are more relevant models than engineered NIH 3T3 cells. Endothelial cells are known to express VEGF receptors (19, 20), and their proliferation in response to VEGF was inhibited by an antibody against Flk-1/KDR (34). We confirmed the presence of VEGF receptors on our endothelial cells by Western blotting. VEGF-treated cells gave a band of the appropriate molecular weight when blotted with an antiphosphotyrosine antibody (data not shown). Furthermore, endothelial cells incubated with VEGF incorporated 5–10-fold more thymidine into DNA than unstimulated cells, indicating that their signaling mechanism from KDR was intact. All compounds found to inhibit Flk-1 kinase also inhibited thymidine incorporation into the DNA of endothelial cells with comparable IC$_{50}$ values (Table 1). This supports the assertion that inhibition of Flk-1/KDR prevents other functions of endothelial cells.
The Flk-1 inhibitors were also evaluated for their effects on angiogenesis in developing CAMs of chicken embryos (39). This system has been used extensively to analyze stimulators and inhibitors of angiogenesis (40). VEGF has been shown to stimulate angiogenesis in CAMs (41). Flk-1 inhibitors were used to treat CAMs of embryos from days 4 to 10 or 11 of incubation, a period when angiogenesis actively occurs. Two of the compounds tested, SU1433 and SU1498, prevented blood vessel formation under these conditions (Fig. 3). Thus, blockers of signal transduction from Flk-1 prevent angiogenesis in an in vivo model.

The same two compounds also inhibited the action of VEGF in a vessel permeability model in which the compounds were implanted into athymic mice along with time-release VEGF pellets (Fig. 5). The vascular permeability function of VEGF has been implicated in the hyperpermeability of blood vessels associated with tumors (21). Although this model does not involve tumors, VEGF clearly caused an effect that was prevented by Flk-1 inhibitors.

Angiogenesis occurs in many disease states, such as tumor growth, psoriasis, and diabetic retinopathy. VEGF and its receptor Flk-1/KDR appear to play a role in angiogenesis in these diseases. Thus, small molecule inhibitors such as the compounds identified in our drug discovery program may be useful therapeutics for a variety of conditions.

ACKNOWLEDGMENTS

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