A Novel Orally Bioavailable Inhibitor of Kinase Insert Domain-Containing Receptor Induces Antiangiogenic Effects and Prevents Tumor Growth in Vivo

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ABSTRACT

A strategy for antagonizing vascular endothelial growth factor (VEGF)-induced angiogenesis is to inhibit the kinase activity of its receptor, kinase insert domain-containing receptor (KDR), the first committed and perhaps the last unique step in the VEGF signaling cascade. We synthesized a novel ATP-competitive KDR tyrosine kinase inhibitor that potently suppresses human and mouse KDR activity in enzyme (IC_{50} = 7.8–19.5 nM) and cell-based assays (IC_{50} = 8 nM). The compound was bioavailable in vivo, leading to a dose-dependent decrease in basal- and VEGF-stimulated KDR tyrosine phosphorylation in lungs from naïve and tumor-bearing mice (IC_{50} = 23 nM). Pharmacokinetics and pharmacodynamics guided drug dose selection for antitumor efficacy studies. HT1080 nude mice xenografts were treated orally twice daily with vehicle, or 33 or 133 mg/kg of compound. These doses afforded trough plasma concentrations approximately equal to the IC_{50} for inhibition of KDR autophosphorylation in vivo for the 33 mg/kg group, and higher than the IC_{50} for the 133 mg/kg group. Chronic treatment at these doses was well-tolerated and resulted in dose-dependent inhibition of tumor growth, decreased tumor vascularization, decreased proliferation, and enhanced cell death. Antitumor efficacy correlated with inhibition of KDR tyrosine phosphorylation in the tumor, as well as in a surrogate tissue (lung). Pharmacokinetics and pharmacodynamics assessment indicated that the degree of tumor growth inhibition correlated directly with the extent of inhibition of KDR tyrosine phosphorylation in tumor or lung at trough. These observations highlight the need to design antiangiogenic drug regimens to ensure constant target suppression and to take advantage of PD end points to guide dose selection.

INTRODUCTION

Ablative angiogenesis is associated with various pathological states such as cancer, neovascular ocular diseases, and inflammation, contributing to disease progression and symptomatology. Neovascularization occurs largely by the cooption and sprouting of new capillaries from the existing microvasculature, involving the proliferation and migration of vascular endothelial cells (ECs), and by the recruitment of EC progenitors from the bone marrow (1, 2). Vascular endothelial growth factors (VEGFs) are key effectors of this process, stimulating proliferation, survival, and migration of ECs (3, 4). VEGF mediates these activities primarily by binding and activating the VEGF receptor-2 or kinase insert domain-containing receptor (KDR, or fetal liver kinase-1 in mice; Refs. 5–7).

In solid tumors the expression of VEGF can be induced by hypoxia, by a variety of growth factors, cytokines, and hormones, by onco- genes, and by the loss of tumor suppressor genes (3, 8). In addition, tumor-infiltrating leukocytes and platelets can also release VEGF on degranulation (3). Most solid tumors are dependent on elevated VEGF expression for angiogenesis. In addition, tumor vascularity and/or plasma VEGF levels have been reported to have independent prognostic value for disease progression and/or overall survival in the majority of human solid tumors and in some hematopoietic malignancies (9–12).

Homozygous and heterozygous murine gene knockouts of KDR or VEGF result in embryonic lethality, in which embryos display very few and disorganized vascular ECs (13–16). The fact that such a severe phenotype is observed even in heterozygotes highlights the importance of the KDR-VEGF axis for vascular development. On the basis of these observations, a number of antagonists and inhibitors of this axis have been developed and are at various stages of preclinical and clinical development (17, 18). Herein we report a proof-of-concept study with a member of a series of indolinone tyrosine kinase inhibitors of KDR. The compound was well tolerated and had antitumor efficacy in a tumor xenograft model in nude mice at doses that inhibited the biochemical target (KDR) and caused cell biological changes consistent with an antiangiogenic effect.

MATERIALS AND METHODS

KDR Kinase Inhibitor. 3-(5-{3-[2-Methoxyethyl(methyl)amino]ethoxy}]-1H-indol-2-yl)-2-(1H)-quinoxaline was prepared in a six-step synthesis from commercially available 5-hydroxyindole (19). Concisely, silylation (TBSiCl) of 5-hydroxyindole and subsequent N-Boc protection provided tert-buty1 5-[(tert-buty1(dimethyl)silyl)oxy]-1H-indole-1-carboxylate. Lithiation followed by the addition of trimethylaluminate gave 1-(tert-buty1(dimethyl)silyl)oxy]-1H-indole-1-carboxylate. Distillation (3HF-Et3N) then afforded tert-buty1 2-(2-chloro-3-quinolinyl)-5-hydroxy-1H-indole-1-carboxylate. Alkylation with 2-chloro-N-(2-methoxyethyl)-N-methylethanamine and cesium carbonate as base followed by hydrolysis (aqueous AcOH) gave the final product as a yellow solid (19). Detailed description of the biochemical and cellular characterization of the compound and corresponding assays is being reported elsewhere.

Tumor Cell Line. HT1080 human fibrosarcoma cells were obtained from and propagated as recommended by the American Type Culture Collection (Manassas, VA). Before in vivo studies, HT1080 cultures were shown to be free of murine pathogens (Infectious Microbe PCR Amplification Testing, University of Missouri Research Animal Diagnostic and Investigative Laboratory) and not to be sensitive to nonspecific cytotoxic effects by the inhibitor. For this purpose cells were seeded to 10^3 cells/100 µl/well on a 96-well plate and allowed to adhere overnight. The following day, the medium was replaced with an equal volume of drug-containing medium, or DMSO, in triplicate using half-log dilutions. After a 48 h incubation at 37°C, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 5 mg/ml in PBS was added to each well, and plates were incubated for an additional 4 h at 37°C. Formazan crystals were extracted in 100 µl of isopropanol and product was quantitated by measuring absorbance at 570 nm. Percentage of cytotoxicity was determined as = [(A_{570control}−A_{570sample})/A_{570control}]×100, and inflection points in dose response curves were reported as IC_{50}.

Animal Studies. Experiments were conducted in accordance with the standards established by the United States Animal Welfare Acts, set up in

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Merck’s Institutional Animal Care and Use Committee. Nude *nu/nu* female mice (Charles River) used in the study were 6–12 weeks of age with average body weight of approximately 25–30 g.

**Lung KDR Autophosphorylation Pharmacodynamic Assay.** The assay protocol will be reported in detailed elsewhere. Briefly, compound was administered at several dose levels either by i.p. injection or p.o. gavage. At various times (1–24 h) after dosing, KDR autophosphorylation was stimulated by a tail-vein injection of human VEGF₁₆₅ 5 min before sacrifice. Blood samples were taken by cardiac puncture to determine compound concentrations in plasma by liquid chromatography (LC)-tandem mass spectrometry. The lungs were quickly removed and stored in liquid nitrogen until processed. Frozen tissue was weighed, pulverized in liquid nitrogen, lysed in detergent-containing buffer, and cleared by centrifugation. The extent of KDR tyrosine phosphorylation was analyzed by immunoprecipitation and immunoblotting with specific antibodies (anti-KDR; SC-504, Santa Cruz Biotechnology and antiphosphotyrosine antibody; 20–321, Upstate Biotechnology). Membranes were then stripped and reprobed with anti-KDR antibody (SC-6251; Santa Cruz Biotechnology) and the ratio of phosphorylated KDR:total KDR signals was calculated and expressed as a percentage of VEGF-stimulated vehicle control-treated mice.

**Tumor Studies.** Five million human HT1080 fibrosarcoma cells in 500 μl of cold medium salt solution were injected s.c. into the left flank of nude mice on day 0. Mice were randomized according to body weight into three treatment groups: (a) 0.5% methylcellulose vehicle (15 mice); (b) KDR kinase inhibitor at 33 mg/kg (mpk) per dose (24 mice); and (c) KDR kinase inhibitor at 133 mg pk per dose (24 mice). Dosing was twice daily and began on day 1 by oral gavage using an administration volume of 5 ml/kg of drug. Body weights and tumor volumes (by caliper) were taken every 2–3 days during the study and at termination. Tumor length and width were used to calculate volume as Width×Width×Length×0.5.

** Necropsy and Tissue Collection.** Animals were sacrificed by CO₂ asphyxiation, and blood was drawn from the inferior vena cava. Plasma was separated by centrifugation at 2600 × g for 20 min and stored at −20°C before pharmacokinetic analyses. Animals were sacrificed at 1, 6, and 12 h after the first and last dose using 3 mice/time point/treatment group to evaluate pharmacokinetic-pharmacodynamic relationships. In addition, representative animals from each group received a tail-vein injection of human VEGF₁₆₅ 5 min before death. Tumors were excised, weighed, and either snap-frozen in liquid nitrogen for determination of tumor KDR phosphorylation as described above or fixed in Zn-Tris for histology. Lungs were frozen immediately in liquid nitrogen for determination of KDR phosphorylation.

**Pharmacokinetic Analysis.** The KDR kinase inhibitor was isolated from mouse plasma by a standard protein precipitation procedure. For the purposes of drug quantitation, a LC system consisting of a Hewlett Packard Series 1100 (Palo Alto, CA) and a Leap Technologies CTC PAL Autosampler (Carboyn, NC) equipped with a 50 μl loop was coupled to an API 2000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Toronto, Ontario, Canada). Compound and an internal standard were separated by reversed-phase high-performance liquid chromatography, and mass spectral analysis was performed on the split-less effluent flow with an Applied Biosystems API 2000 triple-stage quadrupole mass spectrometer equipped with a heated nebulizer ionization source. Data were acquired and analyzed using Sciei Analyst software in selective multiple reaction monitoring mode alternating the following transitions: KDR kinase inhibitor (M+H)⁺ = 392→116 and standard (M+H)⁺ = 405→84. Plasma concentrations were determined by interpolation from a standard curve (lower limit of quantitation = 5 ng/ml). Non compartmental pharmacokinetic parameters were calculated using Watson DMLIMS (Wayne, PA), and area under plasma concentration-time curve was determined by linear-log trapezoidal interpolation.

**Immunohistochemistry.** Paraffin-embedded adjacent central tumor sections (5 μm in thickness) were stained with: (a) H&E to determine tumor morphology; (b) EC marker (CD31); (c) a marker of proliferation (Ki67); and (d) cell death [activated caspase-3 and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL)]. Sections were deparaffinized and hydrated, endogenous peroxidase activity was blocked with 3% H₂O₂, and sections were either treated with Protein Block solution (Biogenex) for CD31 stain or incubated with Target Retrieval Solution (DAKO; S1699) and microwaved for 8–10 min at power level 2 (GE Profile JE1360WB) for Ki67 and activated caspase-3 staining. For CD31, staining sections were incubated in murine monoclonal antirat CD31 (1:400, MA-3107P; Endogen) followed by biotinylated goat antirat IgG (DAKO; K0609) and peroxidase-conjugated streptavidin (DAKO; K0609), and developed with DAB/Nicil2 (KPL Labs; 54–75–00). For Ki67 and activated caspase 3 the reagents used were anti-Ki67 polyclonal antibody NCL-Ki67p at 1:800 (Novacastra) and antiactiavated Caspase 3 polyclonal antibody, 1:3000 (Merek Frssto). PBS/0.3% Tween 20 was used for washes. TUNEL staining was performed using in situ Apoptosis Detection kit, TACS 2 TdT-Blue Label ( Trevigen; 4811–30-K) according to the manufacturer’s instructions.

**Image Analysis.** Stained slides were analyzed and quantitated using a microscope with an automated stage linked to Image-Pro software. H&E stained slides served as guides to delineate the edge of the tumor and other histological features. Data are expressed as the percentage of area stained compared with vehicle controls. Ki67, activated caspase-3, and CD31 stains were analyzed using a Leica DMLB microscope, Optronics DE1750 camera, Prior automated stage, and autofocus. Automated scans of whole tumor central sections were taken with a ×20 magnification objective. TUNEL sections were analyzed on an Olympus microscope, Olympus Magnifier color digital camera. LuliR automated stage, and autofocus, and automated scans of whole tumor central sections were taken with a ×10 objective. Individual files were stored for analysis, and montages were assembled resulting in images of entire sections. Calibration was checked using an image analysis calibration slide. Quantification was done by computer-based image analysis using Image-Pro software (MediaCybernetics). The criteria for quantitation were specific staining and morphological features. Color images were changed to 8-bit gray scale images, and a threshold range was initially established by scanning vehicle and treated tumors for intensity of stain. A numerical range was applied to sequential files, taking into account uneven stain. The files from ×10 and ×20 fields were quantitated as area and percentage area of stained marker. The results were collected and analyzed in Excel. Macros were written to process large batches of files gathered from the tumor sections. An area of interest was defined to eliminate obvious areas of necrosis, except for TUNEL. Whole montages of vehicle- and inhibitor-treated tumors were referenced, showing patterns of growth and inhibition, and also comparisons of stain and morphology.

**RESULTS**

**Activity and Selectivity Profile of the KDR Kinase Inhibitor in Vitro.** The indolyl quinolinone disclosed here, 3-[(5-[(2-methoxyethyl)-(methyl)amino]ethoxy)-1H-indol-2-yl)-2(1H)-quinolinone (Fig. 1), is a potent ATP-competitive inhibitor of human and murine KDR (fetal liver kinase-1) tyrosine kinase activity in vitro, as well as in cell culture (Ref. 19; Table 1). The compound is comparably potent (0.8–2-fold) against the Fli-4, Kit, Fli-3, and platelet-derived growth factor receptor-β kinases, and is 6- and 14-fold selective against Fms and Fli-1, respectively (Table 1). However, the inhibitor is moderately selective (10–100-fold) against most other closely related tyrosine kinases and is highly selective (>1000-fold) against more distantly related kinases. This selectivity was similarly observed in cell-based assays, with potent inhibition of receptor phosphorylation and biological responses observed only on cells expressing and being dependent on sensitive kinases. For example, incubation of two human tumor cell lines (HT1080 fibrosarcoma and LS174T colon carci-

**Fig. 1.** Chemical structure of the kinase insert domain-containing receptor kinase inhibitor 3-[(5-[(2-methoxyethyl)-(methyl)amino]ethoxy)-1H-indol-2-yl)-2(1H)-quinolinone.
Table 1: Activity and selectivity profile of Merck’s KDR inhibitor

<table>
<thead>
<tr>
<th>Assay</th>
<th>IC₅₀ (nM) ± SE</th>
<th>Fold selectivity over human KDR</th>
</tr>
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<tbody>
<tr>
<td>Mouse Flt-1 (enzyme assay in vitro)</td>
<td>19.5 ± 2.7</td>
<td>1</td>
</tr>
<tr>
<td>Human KDR (enzyme assay in vitro)</td>
<td>7.8 ± 1.0</td>
<td>1</td>
</tr>
<tr>
<td>Human KDR phosphorylation and mitogenesis (cell assays)</td>
<td>7.6 to 9.3</td>
<td>1</td>
</tr>
<tr>
<td>Human PDGFR-β, Flt-3, Flt-4, Kit, Fms</td>
<td>0.8-2.0</td>
<td>1</td>
</tr>
<tr>
<td>Human Flt-1 enzyme</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Human Src, FGFR 1 and 2 enzyme</td>
<td>20-60</td>
<td>1</td>
</tr>
<tr>
<td>Human insulin-like growth factor receptor 1, insulin receptor, epidermal growth factor receptor, Tie2 enzyme</td>
<td>1100-2700</td>
<td>1</td>
</tr>
</tbody>
</table>

α KDR, kinase insert domain-containing receptor; PDGFR, platelet-derived growth factor receptor; FGFR, fibroblast growth factor receptor.

β Kinase domain-GST fusion protein (manuscript in preparation).

noma) and human embryonic kidney-293 cells, none of which express KDR, with the compound for 48 h resulted in very low nonmechanism-based toxicity. The IC₅₀ value for inhibition of HT1080 growth after 48-h exposure to drug was 11.1 ± 1.4 μM (n = 4) as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. This concentration is ~700 times higher than what is required to inhibit EC proliferation (Table 1). This result suggests that any antitumor activity observed in vivo against HT1080 tumors would be due to an antiangiogenic effect rather than direct antiproliferative activity against the tumor cell itself.

Antitumor Efficacy and Pharmacodynamic Evaluation. A pharmacodynamic assay was developed to assess whether the achieved plasma concentrations of KDR inhibitor were able to inhibit the biochemical target. The development and validation of this assay are to be described elsewhere, but briefly the assay measures KDR autophosphorylation state in the presence and absence of administered compound. KDR is isolated from either lung or tumor tissues by immunoprecipitation, and then both KDR autophosphorylation state in the presence and absence of administered compound. KDR is isolated from either lung or tumor tissues by immunoprecipitation, and then both KDR autophosphorylation state and total KDR are detected by Western blotting followed by quantitative densitometry. In this assay the KDR inhibitor exhibits an IC₅₀ of 23 nM as measured in lungs of nude mice (data not shown). The results of the assay guided us in the design of an efficacy experiment to choose doses of KDR inhibitor that would be predicted to give >90% inhibition of KDR phosphorylation as well as a dose that would give an intermediate inhibition.

Pilot pharmacokinetics studies in mice demonstrated that oral doses of 33 and 133 mg/kg of the KDR kinase inhibitor administered twice daily would afford the desired plasma levels to inhibit KDR phosphorylation (partial and complete, respectively) and be tolerated by the animals (data not shown). The efficacy study was performed using human HT1080 fibrosarcoma flank tumors implanted in nude mice. HT1080 cells secrete VEGF, do not express KDR, and are not significantly inhibited by the compound directly. We had shown previously that HT1080 tumor growth formation in vivo is dependent on angiogenesis. Forced expression of native soluble Flt-1 in HT1080 cells resulted in VEGF sequestration, and impaired tumor growth and angiogenesis in vivo without affecting the proliferative rate of the tumor cells themselves in tissue culture (20). Moreover, similar results were obtained when treating HT1080 tumor-bearing animals with a blocking antibody against human VEGF.

HT1080 fibrosarcoma cells were injected s.c. into the flank of nude mice, and dosing was initiated the following day, ~16 h after tumor cell implantation (Fig. 2A). Treatment with the KDR kinase inhibitor resulted in profound inhibition of tumor growth (Table 2; Fig. 2).

Tumor growth decreased in a dose-dependent manner in drug-treated animals, with 66.8% and 92% reduction in average terminal tumor mass for the 33 and 133 mpk-treated mice relative to vehicle controls, respectively (Fig. 2). In fact, the 92% inhibition of tumor mass (i.e., 8% of vehicle control) in the 133 mpk dose group appears to underestimate the inhibition of the receptor on the basis of histology that revealed most of the excised sample mass to contain host tissue. Although no compound-related loss in total body weight was observed in any group (Table 2), 2 mice died in the high-dose group, as well as 1 each in the low-dose and vehicle groups, which may have been the result of dosing trauma.

Pharmacokinetics were evaluated from plasma samples obtained from 3 mice per time point for each group at 1, 6, and 12 h after the first dose on day 1, and at similar time points after the last dose. Average plasma drug concentrations are shown in Fig. 2C. Although a 1.8–9-fold increase in drug exposure was observed at 1–6 h after repeated dosing (day 11 versus day 1), 12-h trough plasma concentrations were not significantly different. Doses of 33 and 133 mg/kg administered twice daily resulted in area under the curve (AUC) values of 16,620 and 89,809 ng h, respectively, and afforded 12-h trough levels of 19 and 2,200 ng (Table 2; Fig. 2C). Trough plasma values were in agreement with the targeted values of 23 nM and 1,900 nM, the IC₅₀ and estimated IC₅₀ for inhibition of KDR tyrosine phosphorylation in vivo described above.

Pharmacodynamic assessment of drug action was determined at termination by measuring the levels of basal and VEGF-stimulated KDR tyrosine phosphorylation in lungs and tumors, and by evaluating the degree of tumor vascularity, proliferation, and cell death by immunohistochemical analysis of tumor sections (Fig. 2D; Fig. 3). Some animals received exogenous stimulation with human VEGF 5 min before sacrifice. Drug treatment resulted in partial (55.8–75.2%) and nearly full (96.7–99.3%) inhibition of KDR phosphorylation in lung and tumor lysates from the tumor-bearing animals, in full agreement with lung phospho-KDR measurements in naive animals (IC₅₀ = 23 nM). Exogenous VEGF stimulation did not affect the extent of receptor inhibition, or additionally increase the level of KDR tyrosine phosphorylation, which was already elevated, possibly due to systemic exposure to tumor-derived VEGF (Table 2; data not shown).

Histochemical analysis of the vehicle-treated animal demonstrated circumscribed encapsulated masses composed of solid sheets of pleomorphic cells. The cells had small to moderate amounts of eosinophilic granular cytoplasm and centrally located rounded nuclei with finely granular chromatin. Stromal elements were minimal, and occasional foci of caseous necrosis were noted. Treatment with inhibitor resulted in dose-dependent moderate to severe centralized necrosis, rimmed by a leading edge of viable tumor cells interspersed with a loosely woven fibrovascular stroma. Parallel tumor sections were stained with specific markers for tumor growth (Ki67), cell death (activated caspase 3, and TUNEL), and vascularity (CD31; Fig. 3). Representative ×20 fields and whole tumor images shown in Fig. 3, A and B, reveal an inverse pattern for proliferative and cell death markers. At the 33 and 133 mpk doses, respectively, we observed a 78% and 98% decrease in vascularity, 36% and 64% decrease in proliferation, and >220% increase in cell death. These results correlate the observed inhibition of lung and tumor KDR phosphorylation (Table 2; Fig. 2D). Tumor vascularity was widespread in control tumors, with “hot spots” clearly visible in the periphery of the bilobular control tumor. Treated tumors exhibited a notable dose-dependent suppression of vascularity. Tumors from the 133 mpk-cohort were clearly necrotic, with a poorly vascularized thin viable rim, which included surrounding normal tissue and skin (Fig. 3B).
Fig. 2. In vivo effects of kinase insert domain-containing receptor (KDR) kinase inhibitor: antitu-
mor efficacy and pharmacodynamic evaluation. A, study design. HT1080 human fibrosarcoma cells were implanted s.c. into the flanks of nude mice on day 0. The following day, twice-daily oral dosing began and continued daily until termination on day 11, after 10.5 days of treatment. B, tumor volumes were estimated during the course of the study from caliper measurements of tumor dimensions using the formula width/2 x length. Graph shows means of tumor volumes; bar, ± SE. C, pharmaco-
kinetin profile of KDR kinase inhibitor on the first and last day of dosing. Drug concentrations (Cp) were determined on plasma samples obtained at 1, 6, or 12 h, after the first (open symbols) and last (closed symbols) doses. Graph shows means of Cp from at least 3 animals/dose; bars, ± SE. D, pharmaco-
dynamic effects. The extent of KDR tyrosine phosphorylation (KDR autophosphorylation state) was determined by immunoprecipitation/im-
munoblotting from protein extracts from tumors and lung samples obtained from the animals on the study. Phospho-KDR levels were normalized to KDR protein and expressed as a percentage of vehicle. E, terminal weights were determined on freshly collected tumors. The histogram represents means of tumor weights; bars, ± SE. Statistical differences between groups were determined using Student’s t test.

**DISCUSSION**

Numerous preclinical efficacy studies have been reported using small molecule inhibitors of the VEGF-KDR pathway and antibodies to both the growth factor and receptor (17). Herein we describe a novel KDR kinase inhibitor, and link antitumor activity with its in vivo biochemistry and cell biology. At the doses administered, the inhibitor was well tolerated, and afforded drug plasma exposures that led to near complete and complete target suppression at the low and high dose, respectively, as determined by pharmacokinetic-pharma-
codynamic assessment. It is interesting to note that the tumor growth inhibition correlated directly with: (a) plasma drug concentration; and (b) the degree of target inhibition in the tumor and lungs obtained at the 12-h trough. Pharmacodynamic analysis was extended further to include histological assessment of angiogenesis, and tumor proliferation and survival (Fig. 3). Vascular EC density and cellular prolif-
eration decreased as a percentage of each tumor section as a function of dose. In contrast, cellular death within tumors, attributable to apoptosis and necrosis, increased as a function of dose.

These results are consistent with a mechanism in which the compound inhibits KDR-dependent tumor angiogenesis, resulting in de-
creased proliferation and increased cellular death within tumors at doses that are well tolerated by mice. However, this compound is almost equipotent against Flt-4 and platelet-derived growth factor receptor-β, and is only 14-fold less potent against Flt-1 (Table 1); therefore, these kinases are also expected to be inhibited to some degree at the exposures attained in vivo and to contribute to the antitumor effects. Although VEGF-activation of Flt-1 is not directly mitogenic to ECs, its activation by placental growth factor results in intermolecular transphosphorylation and activation of KDR (21). Moreover, Flt-1 has been implicated in mediating the recruitment of EC progenitors from the bone marrow to areas of active angiogenesis (2, 22). On the other hand, Flt-4 has also been linked to tumor angiogenesis. Elevated expression of its ligands VEGF-C and -D has been correlated with lymph node involvement, metastasis, and poor prognosis in cancer patients (23). Activation of the Flt-4 pathway in preclinical models induced tumor lymphangiogenesis and increased metastatic spread, whereas inhibition of the pathway was able to prevent tumor growth and vascularization (24–26). Finally, tumor vessel stabilization is mediated via interactions with smooth muscle pericytes, involving the platelet-derived growth factor receptor path-

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**Table 2. In vivo anti-tumor efficacy, pharmacokinetics (PK), and pharmacodynamic (PD) correlates for KDR kinase inhibitor in murine HT1080 xenografts**

<table>
<thead>
<tr>
<th>End point</th>
<th>Treatment group</th>
<th>Vehicle</th>
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<tr>
<td>Antitumor efficacy</td>
<td></td>
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<tr>
<td>% Vehicle</td>
<td></td>
<td>100.0 ± 17.7</td>
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<td>% Inhibition</td>
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<tr>
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<td>−3.7%</td>
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<tr>
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<td>AUC(1−2) (min hr)</td>
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<td>Cp at 12 h (hr)</td>
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<td>218</td>
<td>287</td>
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* KDR, kinase insert-domain containing receptor; VEGF, vascular endothelial growth factor; IHC, immunohistochemistry; AUC, area under the concentration-time curve; TUNEL, terminal deoxynucleotidyl transfrerase-mediated nick end labeling; mpk, ng per kg.

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Inhibitor of KDR induces antiangiogenic effects

Preclinical evidence supports the premise that inhibiting multiple targets could lead to more profound antiangiogenic effects. Combining blocking antibodies against KDR and Flt-1 have synergistic effects compared with the effect of each antibody given singly (22). Treatment of RIP-TAg mice with a combination of KDR and platelet-derived growth factor receptor kinase inhibitors, either as drug combinations (SU5416 and Gleevec) or using a multitarget inhibitor (SU6668), resulted in a synergistic decrease in the development of pancreatic islet tumors, in tumor angiogenesis, and in regression of established tumors (29). Similar observations have been made with other multitarget inhibitors (30–32). Thus, affecting the interaction of the endothelium with the supporting pericytes and stromal elements may be a desired add-on feature for antiangiogenic agents, such as the one described here. This premise is being tested in the clinic with small molecule inhibitors such as PTK-787, SU11248, ZD6474, and CEP-7055 (18), which exhibit diverse selectivity profiles, and by bevacizumab (Avastin; Genentech, Inc.), a VEGF neutralizing monoclonal antibody that blocks activation of both KDR and Flt-1. In fact, bevacizumab was shown recently to have clinical activity in renal cell carcinoma and colon cancer (33, 34).

In summary, we introduced a novel KDR kinase inhibitor, and demonstrated molecular and preclinical efficacy in a murine system. We used a pharmacokinetic-pharmacodynamic approach for dose selection and demonstrated that inhibition of KDR phosphorylation in lung tissue from naïve animals is predictive of target modulation in the tumor tissue. Moreover, we established that tumor growth inhibition is correlated directly with the extent of KDR inhibition observed at trough, when drug concentrations are the lowest. This observation gives support to the necessity for prolonged target suppression to achieve clinically efficacious responses in tumor growth.

REFERENCES

INHIBITOR OF KDR INDUCES ANTIANGIOGENIC EFFECTS

A Novel Orally Bioavailable Inhibitor of Kinase Insert Domain-Containing Receptor Induces Antiangiogenic Effects and Prevents Tumor Growth in Vivo

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