KRN951, a Highly Potent Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases, Has Antitumor Activities and Affects Functional Vascular Properties

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Abstract

Vascular endothelial growth factor (VEGF) plays a key role in tumor angiogenesis by stimulating the proangiogenic signaling of endothelial cells via activation of VEGF receptor (VEGFR) tyrosine kinases. Therefore, VEGFRs are an attractive therapeutic target for cancer treatment. In the present study, we show that a quinoline-urea derivative, KRN951, is a novel tyrosine kinase inhibitor for VEGFRs with antitumor angiogenesis and antigrowth activities. KRN951 potently inhibited VEGF-induced VEGFR-2 phosphorylation in endothelial cells at in vitro subnanomolar IC_{50} values (IC_{50} = 0.16 nmol/L). It also inhibited ligand-induced phosphorylation of platelet-derived growth factor receptor-β (PDGFR-β) and c-Kit (IC_{50} = 1.72 and 1.63 nmol/L, respectively). KRN951 blocked VEGF-dependent, but not VEGF-independent, activation of mitogen-activated protein kinases and proliferation of endothelial cells. In addition, it inhibited VEGF-mediated migration of human umbilical vein endothelial cells. Following p.o. administration to athymic rats, KRN951 decreased the microvessel density within tumor xenografts and attenuated VEGF-2 phosphorylation levels in tumor endothelium. It also displayed antitumor activity against a wide variety of human tumor xenografts, including lung, breast, colon, ovarian, pancreas, and prostate cancer. Furthermore, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) analysis revealed that a significant reduction in tumor vascular hyperpermeability was closely associated with the antitumor activity of KRN951. These findings suggest that KRN951 is a highly potent, p.o. active antiangiogenesis and antitumor agent and that DCE-MRI would be useful in assessing treatment responses. KRN951 is currently in phase I clinical development for the treatment of patients with advanced cancer.

Introduction

Vascular endothelial growth factor (VEGF) is involved in tumor angiogenesis and plays an important role in tumor malignancy, such as sustaining tumor growth and in blood-borne metastasis. VEGF regulates both vascular growth and permeability, and it also functions as an antiapoptotic factor for newly formed vessels (1). VEGF expression is up-regulated by changes associated with cancer, such as hypoxia, proto-oncogene activation, loss of tumor suppressor gene expression, and growth factor stimuli in tumors (1, 2). Its overexpression has been reported to correlate with the degree of vascularity, poor prognosis, and aggressive disease in the majority of human solid tumors (3–6) and in some hematopoietic malignancies (7).

Two high-affinity cognate endothelial receptors for VEGF have been identified (8, 9): VEGF receptor-1 (VEGFR-1; also known as Flt-1) and VEGFR-2 [also known as kinase insert domain-containing receptor (KDR)/Flk-1]. Both are members of a large family of receptor tyrosine kinases, and are almost exclusively located in endothelial cells. Activation of VEGFR-1 and VEGFR-2 receptors occurs through VEGF binding, which triggers receptor dimerization, tyrosine kinase activation, and phosphorylation of tyrosine residues. Although the affinity of VEGF for VEGFR-1 is higher than for VEGFR-2, the major mitogenic, angiogenic, and permeability-enhancing effects of VEGF seem to be mediated through VEGFR-2.

Several experimental approaches aimed at blocking VEGF signaling have shown that VEGF and its receptors are not only essential molecules for tumor angiogenesis, but are also attractive targets for cancer therapy (10). These approaches included the use of VEGF-neutralizing monoclonal antibodies, antibodies against VEGF receptors, recombinant soluble receptors, a tetracycline-regulated VEGF expression system, a dominant-negative VEGF receptor mutant, and small-molecule inhibitors of VEGFR tyrosine kinases. Among these, antibodies and small molecules were further developed as practical agents for cancer treatment both in preclinical and clinical settings (11). In particular, bevacizumab, a recombinant humanized monoclonal antibody to VEGE, has been approved as a first-line therapy for metastatic colorectal cancer (12). In addition, several VEGF tyrosine kinase inhibitors are currently under preclinical or clinical development (13–21). Recently, two small-molecule inhibitors with activities against VEGF tyrosine kinase, BAY 43-9006 (17) and SU11248 (14), have been approved for the treatment of patients with advanced renal cell cancer.

In the present study, we describe a novel quinoline-urea derivative, which is a highly potent VEGF tyrosine kinase inhibitor, and also highlight the potential of using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) to assess treatment responses.

Materials and Methods

KRN951. N-{2-Chloro-4-[(6,7-dimethoxy-4-quinoxllyl)oxy]phenyl}-N’-(5-methyl-3-isoxazolyl) urea hydrochloride monohydrate (KRN951; Fig. 1)
was synthesized in the Production Department, Research and Development Center, Kirin Brewery, Co., Ltd. (Tokyo, Japan). For in vitro studies, KRN951 was dissolved in DMSO and diluted in growth medium immediately before use. For in vivo studies, it was suspended in vehicle (0.5% methylcellulose in distilled water) and the suspension was administered to animals within a day of preparation.

Cell lines. Human umbilical vein endothelial cells (HUVEC) and normal human dermal fibroblasts were obtained from Cambrex (Walkersville, MD). VEGFR-1–overexpressing NIH3T3 cells, designated NIH3T3-Flt-1, have been described previously (22). Colo205 (human colon carcinoma) and KU812-F (human chronic myelogenous leukemia) cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The A431 (human epidermoid carcinoma) cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan). A549 (human lung carcinoma) and EoL-1 (human eosinophilic leukemia) cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The CGL-9 (human glioma) cell line was obtained by mechanical dissociation of a s.c. SNB 19 (human glioma) tumor xenografted in athymic rats. All other cell lines were purchased from the American Type Culture Collection (Manassas, VA) unless otherwise specified.

Kinase selectivity. Cell-free kinase assays were done in quadruplicate with 1 μmol/L ATP to determine the IC₅₀ values of KRN951 against a variety of recombinant receptor and nonreceptor tyrosine kinases. Recombinant enzymes were obtained from ProQinase GmbH (Freiburg, Germany).

Cell-based assays were done to determine the ability of KRN951 to inhibit ligand-dependent phosphorylation of receptor tyrosine kinases as described previously (19). Briefly, the cells were starved overnight in appropriate basic medium containing 0.5% fetal bovine serum (FBS). Following the addition of KRN951 or 0.1% DMSO, the cells were incubated for 1 hour and then stimulated with the cognate ligand at 37°C. Receptor phosphorylation was induced for 5 minutes except for VEGFR-3 (10 minutes), c-Met (10 minutes), and c-Kit (15 minutes). All the ligands used in the assays were human recombinant proteins, except for VEGF-C, a rat recombinant protein. Following cell lysis, receptors were immunoprecipitated with appropriate antibodies and subjected to immunoblotting with phosphotyrosine. Quantification of the blots and calculation of IC₅₀ values were carried out as described previously (19).

Mitogen-activated protein kinase activation. This was evaluated as described previously (19). Briefly, HUVECs were starved for 16 hours in a basic medium (EBM-2, Cambrex) containing 0.5% FBS. Following incubation with KRN951 for 1 hour, HUVEC stimulated with 50 ng/mL VEGF (PeproTech EC, Ltd., London, United Kingdom), 25 ng/mL basic fibroblast growth factor (bFGF; Upstate Biotechnology, Charlottesville, VA) or 20 ng/mL EGF (PeproTech EC). Cell lysates were subjected to SDS-PAGE followed by Western blotting with phosphotyrosine.
by immunoblotting of phosphorylated MAPKs with phosphorylated p44/42 mitogen-activated protein kinase (MAPK) antibody (Cell Signaling Technology, Inc., Beverly, MA).

**Endothelial cell proliferation.** HUVECs were seeded in M-199 (Invitrogen, Carlsbad, CA) containing 5% FBS in collagen-coated 96-well plates (BD Biosciences, Bedford, MA) at a density of 4,000 cells/200 µL/well. After 24 hours, KRN951 was added followed by 20 ng/mL VEGF or 10 ng/mL bFGF, and the cells were cultured for 72 hours. [3H]thymidine (1 µCi/mL) was added and the cells were cultured for a further 12 hours. Cells were then harvested and their radioactivity was measured with a Liquid Scintillation Counter (Wallac 1205 Beta Plate; Perkin-Elmer Life Sciences, Boston, MA).

**Chemotaxis assay.** HUVEC migration was assessed using 96-well microculture plates (BD BioCoat Angiogenesis System, BD Biosciences). Cells were starved for 5 hours in EB-2 containing 0.1% bovine serum albumin (BSA). Then, cells were harvested, resuspended in EB-2 containing 0.1% BSA, and placed in the upper chamber. Cell migration was initiated by placing medium containing 10 ng/mL VEGF, 0.1% FBS, and 0.1% BSA to the bottom chamber. When indicated, KRN951 was added to both the upper and lower chambers. After 22 hours of incubation, cells were stained with 4 µg/mL calcein AM in HBSS. Fluorescence in the cells that had migrated through the pores of the fluorescence blocking membrane was directly measured through the bottom of the chambers in a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm.

**Cytotoxicity assays.** These assays were done as described previously (19). Briefly, cells were seeded in 96-well plates and cultured in medium containing 10% FBS. KRN951 was added ~24 hours after the start of culture and the cells were then incubated for 72 hours. WST-1 reagent (Roche Applied Science, Indianapolis, IN) was used for the detection of cell viability.

**Measurement of phosphorylated VEGFR-2 levels and detection of tumor microvessels.** Athymic rats (F344/N Jcl-rnu) were obtained from CLEA Japan, Inc. (Tokyo, Japan). A549 tumor xenografts were established in rats by s.c. implantation of cells. Drug treatments were initiated when tumors of rats by s.c. implantation of cells. Drug treatments were initiated when tumors of rats by s.c. implantation of cells. Drug treatments were initiated when tumors of rats by s.c. implantation of cells. Drug treatments were initiated when tumors of rats by s.c. implantation of cells. Drug treatments were initiated when tumors of rats by s.c. implantation of cells. Drug treatments were initiated when tumors of rats by s.c. implantation of cells. Drug treatments were initiated when tumors of rats by s.c. implantation of cells. Drug treatments were initiated when tumors of rats by s.c. implantation of cells. Drug treatments were initiated when tumors of rats by s.c. implantation of cells. 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**Results**

**KRN951 activity against receptor tyrosine kinases.** The direct activity of KRN951 against various tyrosine kinases was evaluated using a cell-free kinase assay with recombinant enzymes. KRN951 strongly inhibited VEGFR-1, VEGFR-2, and VEGFR-3 tyrosine kinases (IC\textsubscript{50} = 30, 6.5, and 15 nmol/L, respectively) and also inhibited EphB2, PDGFR-\(\alpha\), PDGFR-\(\beta\), c-Kit, and Tie2 tyrosine kinases (IC\textsubscript{50} = 24, 40, 49, 78, and 78 nmol/L, respectively). The level of KRN951 activity against several other tyrosine kinases revealed a more comprehensive selectivity with IC\textsubscript{50} values greater than for VEGFR-2 [e.g., IC\textsubscript{50} = 480 nmol/L for EphB4; 530 nmol/L for FGF-R1: 550 nmol/L for c-Met; 620 nmol/L for Abl; 960 nmol/L for Src; and >1 \(\mu\)mol/L for FGFR-3, FGFR-4, Flt3, EGFR, ErbB2, insulin-R, Fak, ErbB4, insulin-like growth factor (IGF)-1R, and Jak2].

Similarly, KRN951 markedly inhibited the ligand-induced phosphorylation of VEGFR-1, VEGFR-2, and VEGFR-3 in the cellular assay (IC\textsubscript{50} values 0.16-0.24 nmol/L; Table 1). Although KRN951 also inhibited c-Kit and PDGFR-\(\beta\) phosphorylation, the IC\textsubscript{50} values were ~10-fold higher than for VEGF-2. The KRN951 activity level against the phosphorylation of FGF-R1, Flt3, c-Met EGFR, and IGF-IR was considerably lower.

**KRN951 selectively inhibits VEGF signaling responses in endothelial cells.** High concentrations of KRN951 (>0.3 nmol/L) were found to inhibit VEGF-stimulated VEGF-2 phosphorylation (Fig. 1B). Consistent with this, KRN951 strongly inhibited VEGF-dependent phosphorylation of MAPKs in HUVECs (Fig. 1B), demonstrating IC\textsubscript{50} values of 0.13 and 0.18 nmol/L for extracellular signal-regulated kinase 1 (ERK1) and ERK2, respectively. In contrast, KRN951 had little or no effect on bFGF-dependent and EGFR-dependent phosphorylation of MAPKs. As shown in Fig. 1C, KRN951 inhibited the VEGF-induced proliferation of HUVECs at very low concentrations (IC\textsubscript{50} = 0.67 nmol/L). Conversely, the inhibitory activity of KRN951 against bFGF-induced proliferation was weak (IC\textsubscript{50} > 300 nmol/L). Thus, the selectivity of KRN951 for VEGF-induced mitogenic responses, rather than for bFGF- or EGF-induced responses, is consistent with receptor selectivity. In addition, KRN951 at 1 nmol/L reduced the VEGF-mediated migration of HUVECs by ~40% (Fig. 1D). At concentrations of

### Table 1. Effects of KRN951 on the ligand-stimulated phosphorylation of receptor tyrosine kinases

<table>
<thead>
<tr>
<th>RTK</th>
<th>Cell</th>
<th>IC\textsubscript{50} (nmol/L)</th>
<th>95% Confidence interval (nmol/L)</th>
<th>Fold selectivity vs VEGF-2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR-2</td>
<td>HUVEC</td>
<td>0.16</td>
<td>0.13-0.20</td>
<td>1</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>NIH3T3-Flt-1*</td>
<td>0.21</td>
<td>0.16-0.30</td>
<td>1.3</td>
</tr>
<tr>
<td>VEGFR-3</td>
<td>HUVEC</td>
<td>0.24</td>
<td>0.16-0.34</td>
<td>1.5</td>
</tr>
<tr>
<td>c-Kit</td>
<td>KU812F</td>
<td>1.63</td>
<td>1.13-2.35</td>
<td>10</td>
</tr>
<tr>
<td>PDGFR-(\beta*)</td>
<td>NHDF</td>
<td>1.72</td>
<td>1.39-2.13</td>
<td>11</td>
</tr>
<tr>
<td>Flt3</td>
<td>EoI-1</td>
<td>422</td>
<td>342-522</td>
<td>2.640</td>
</tr>
<tr>
<td>FGFRI</td>
<td>NHDF</td>
<td>299</td>
<td>214-417</td>
<td>1.870</td>
</tr>
<tr>
<td>c-Met</td>
<td>A431</td>
<td>1,360</td>
<td>730-2,540</td>
<td>8,500</td>
</tr>
<tr>
<td>EGFR</td>
<td>A431</td>
<td>ND*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>HT29</td>
<td>ND*</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

NOTE: IC\textsubscript{50} values and their 95% confidence intervals were calculated by nonlinear regression analysis of the percentage inhibitions (\(n = 4\)).

Abbreviations: NHDF, normal human dermal fibroblast; RTK, receptor tyrosine kinase; ND, not determined.

*Ratio for the IC\textsubscript{50} was obtained with a given RTK compared with that achieved against VEGFR-2.

1F1-1-transfected NIH3T3.

2IC\textsubscript{50} values could not be determined because KRN951 was apparently insoluble in the medium at the concentrations tested.
≥10 nmol/L, KRN951 completely inhibited VEGF-induced cell migration.

**KRN951 suppresses phosphorylated VEGFR-2 on tumor endothelium and tumor angiogenesis following p.o. administration.** The A549 tumors from control rats that received vehicle only showed colocalization of fluorescent green staining of CD31 with fluorescent red staining of phosphorylated VEGFR-2. In contrast, tumors from rats that received a single dosing of 0.2 mg/kg KRN951 showed apparent attenuation of VEGFR-2 phosphorylation on the tumor endothelium (Fig. 2A). Once-daily p.o. administrations of 0.04, 0.2, or 1.0 mg/kg KRN951 for 7 days resulted in a reduction in the numbers of CD31-positive cells in viable regions of the implanted A549 tumors in athymic rats by 15.7%, 20.7%, and 78.2%, respectively (P < 0.01) compared with vehicle treatment alone (Fig. 2B and C). In addition, H&E staining indicated that KRN951 increased the percentage of necrotic areas within the tumors (data not shown). Tumor growths were almost completely inhibited during the treatment at doses of 0.2 and 1 mg/kg KRN951 (data not shown). These doses resulted in tumor regression of 2% and 33%, respectively.

**Effects of KRN951 on tumor xenograft growth in athymic rats.** The antitumor effects of KRN951 on various human tumors were evaluated in athymic rat xenograft models of breast, colon, hepatic, lung, ovarian, pancreatic, and prostate tumors. The results are summarized in Table 2. After once-daily p.o. administrations of KRN951 for 2 weeks, statistically significant inhibition of tumor growth (P < 0.05) was observed in 10 of 14 models receiving a 0.2 mg/kg dose. A significant and almost complete inhibition of tumor growth (TGI > 85%) was observed in 11 of 14 models receiving a 1.0 mg/kg dose. In addition, tumor regression occurred following treatment with KRN951 at 1.0 mg/kg in the MDA-MB-231 and LoVo models (smallest RTV = 0.89 at day 7 and 0.85 at day 21, respectively). Although KRN951 administration did not produce significant antitumor effects in the DU145, CGL-9, or Caki-1 models because of large variations in tumor volumes of vehicle-treated rats, it nevertheless showed a tendency to inhibit the growth of these tumor xenografts in a dose-dependent manner.

KRN951 also induced a loss of rat body weight following the administration of 1.0 mg/kg dosages in some models; however, these weight losses recovered quickly after completion of administration. No body weight losses were observed at 0.2 mg/kg dosages, suggesting that KRN951 is well tolerated at efficacious dosages.

No substantial effects following treatment with up to a few μmol/L KRN951 were observed on the in vitro growth of cancer cell lines, some of which were used in the xenograft rat models, suggesting that KRN951 is not toxic to cancer cells (data not shown).

**Effects of KRN951 on vascular permeability in Calu-6 tumors: analysis using DCE-MRI.** To evaluate the effects of KRN951 on tumor vascular function and correlate these with its antitumor activities, Calu-6 tumor-bearing rats were subjected to DCE-MRI analysis. The T₂-weighted MRI revealed that tumor growth was markedly inhibited in rats treated with 0.2 or 1.0 mg/kg KRN951 between days 6 and 21 and days 2 and 21, respectively, when compared with vehicle-treated rats (Fig. 3A). The Ktrans values in the rim of the tumors were significantly decreased (by 45%) at day 13 in rats treated with 0.2 mg/kg KRN951, and at day 2 (by 55%) and day 13 (by 61%) in rats treated with 1 mg/kg KRN951, when compared with vehicle-treated rats (Fig. 3B). These
The ratio of enlarged vessels with a diameter of >100 μm increased in tumors from rats treated with 0.2 and 1 mg/kg KRN951 (24.3% and 30.3%, respectively) when compared with rats treated with vehicle (17.5%). In addition, tumor sections from KRN951-treated rats exhibited a greater staining intensity than those from vehicle-treated rats. Pericyte coverage of tumor vessels was further analyzed by dual immunostaining with anti-CD31 antibody and anti-α-smooth muscle actin-antibody, which detect endothelial cells and pericytes, respectively. After 14 days of therapy, the proportion of endothelial cells associated with pericytes increased from 20.7% in the vehicle-treated group to 29.6% in the 0.2 mg/kg KRN951 group and 50.5% in the 1 mg/kg KRN951 group (P < 0.01; Fig. 4C). Simultaneously, treatment with 0.2 and 1 mg/kg KRN951 decreased microvessel density by 42.5% (P < 0.01) and 71.2% (P < 0.001) compared with vehicle, respectively (data not shown).

**Pharmacokinetic profile of p.o. administered KRN951 in athymic rats.** Rat KRN951 serum concentration after p.o. administration was determined to confirm exposure, and pharmacokinetic analysis was done to analyze the correlation with its efficacy (Supplementary Table S1). The area under the serum concentration-time curve from zero to infinity (AUCinf) was 0.34, 1.64, 7.64, and 44.5 μg h/mL and the Cmax was 21.6, 108, 484, and 2,823 ng/mL after a single p.o. administration of 0.04, 0.2, 1, or 5 mg/kg KRN951, respectively. In the 0.04 to 5 mg/kg range, both the AUCinf and Cmax of KRN951 increased in proportion to the dose, whereas p.o. clearance (CL/F), apparent distribution volume (Vd/F), and elimination half-life (t1/2) were similar, suggesting that KRN951 has an approximately linear pharmacokinetic profile in female athymic rats in this dose range. Pharmacokinetic simulation analysis was also carried out in which KRN951 achieved steady state soon after repeated p.o. administration (data not shown). The trough concentration at steady-state (Cmin), the maximum concentration at steady-state (Cmax), and the average concentration at steady-state (Cavg) of 0.2 mg/kg/d KRN951 were simulated to be 22.2, 124, and 69.5 ng/mL, respectively (data not shown).

## Discussion

It is now widely accepted that VEGF signaling through the VEGFR-2 in endothelial cells is primarily responsible for tumor permeability. Athymic rats bearing Calu-6 tumors were randomized at day 14 and treated with 0.2 mg/kg KRN951 (○), 1 mg/kg KRN951 (▲), or vehicle (●) once daily for 14 days (days 0–13). At the days indicated, contrast agent (Gd-DTPA) was injected i.v. before MRI analysis. T2-weighted anatomic and T1-weighted perfusion images were taken to indicate localization and contrast agent uptake of tumors, respectively. A. Tumor volumes were determined by MRI analysis. B. Capillary permeability (Ktrans) in tumors was calculated from perfusion curves, which were generated from ROIs on the periphery of the tumor. P values were calculated by comparing with the vehicle-treated group using the unpaired t test. **, P < 0.01; ***, P < 0.001.
angiogenesis. In this study, we showed the direct activity of KRN951 against VEGFR-2 tyrosine kinase using recombinant proteins, which led to the inhibition of VEGF-stimulated VEGFR-2 phosphorylation in endothelial cells. The selectivity of KRN951 for VEGFR-2 tyrosine kinase was confirmed in both cell-free and cell-based assays. These results are consistent with the ability of KRN951 to inhibit VEGF-driven, but not bFGF- or EGF-driven, phosphorylation of MAPK and cell proliferation. In addition, we showed that VEGF-mediated cell migration, which precedes cell proliferation and is essential for angiogenesis, was inhibited by KRN951. We also showed that p.o. administration of KRN951 significantly reduced microvessel density in tumors and inhibited tumor growth across multiple organ-specific tumor types in rat xenograft models, despite the absence of cytotoxic effects during in vitro cell growth. In a similar in vivo model, we observed a decrease in VEGF-2 phosphorylation on tumor endothelium after KRN951 treatment, indicating that the primary in vivo target of KRN951 is VEGFR-2 phosphorylation. On the other hand, the angiogenic program triggered by VEGFs may involve possible heterodimerization between VEGFR-1, VEGFR-2, and VEGFR-3. The effects of KRN951 on these heterodimer complexes remains to be shown. However, this may not be an important consideration as it seems that the major angiogenic signals elicited by VEGF are mediated by the VEGFR-2 homodimer complex (1). In addition, KRN951 would be expected to act on heterodimer complexes as well, based on its property as a pan-VEGFR tyrosine kinase inhibitor. Accordingly, it is clear that KRN951 is a highly potent VEGFR-2 tyrosine kinase inhibitor with antitumor and antiangiogenesis properties that might be applicable for the treatment of solid tumors and other diseases involving pathologic angiogenesis.

Although many synthetic VEGFR-2 tyrosine kinase inhibitors have been reported (13–21), it is worth noting that KRN951 possesses significantly more potent in vitro and in vivo activities in comparison. KRN951 inhibited VEGF-dependent proliferation of HUVECs with an IC₅₀ of 0.67 nmol/L, whereas in similar assays, PTK787/ZK 222584 (13), SU11248 (14), CP-547,632 (15), and

Figure 4. Effects of KRN951 on tumor vessel diameter and pericyte coverage. Tumor-bearing rats were treated with vehicle or KRN951 for 14 days. Cryosections of the tumor tissues were prepared following i.v. injection of H33342 fluorescent dye for assessment of vessel diameter (n = 3). Paraffin sections were also prepared for assessment of pericyte coverage of tumor vessels (n = 6). A, representative images of tumor sections from rats treated with vehicle, 0.2 mg/kg KRN951, and 1 mg/kg KRN951 were captured under UV illumination (top) or using a fluorescent microscope (bottom) at ×200 magnification for assessment of vessel diameter or pericyte coverage of tumor vessels, respectively. Blue, H33342; green, CD31-positive endothelial cells; red, α-smooth muscle actin-positive pericytes. B, the length of the major axis of the halos related to H33342 (blue fluorescence) was determined by imaging analysis. A column scatter plot shows each value in a group. The superimposed line represents the median and interquartile range of the group. P values were calculated with respect to the control (vehicle) group using the Mann-Whitney test. *, P < 0.05; **, P < 0.01. C, the percentage of pericyte coverage on tumor-associated endothelial cells was determined by counting positive vessels in six fields within each individual tumor and six tumors per group as described Materials and Methods. P values were calculated with respect to the control group using Dunnett's test. **, P < 0.01.
KRN633 (19, 20) showed higher IC_{50} values of 7.1, 4, 14, and 15 nmol/L, respectively. Only AZD2171 (21) shows a comparable level of inhibition (IC_{50} = 0.4 nmol/L).

The strong in vitro activities of KRN951 probably reflect its high activity in vitro. Indeed, we observed severely reduced levels of phosphorylated VEGFR-2 on tumor endothelial cells and significant and broad-spectrum antitumor activities at very low doses (0.2 or 1 mg/kg/d) following single and repeated administration, respectively. In view of this and considering the results of clinical investigations into angiogenesis inhibitors, such as PTK787 (25), pharmacologically active KRN951 serum concentrations are expected to be easily achievable. Moreover, it is likely that KRN951 would produce broader antitumor activities as a pan-VEGFR inhibitor than other compounds, such as specific antibodies targeting the VEGF and VEGFR-2 systems. KRN951 shows almost equipotent in vitro anti-VEGFR-1 and anti-VEGFR-3 tyrosine kinase activities, and it is expected that the extent of in vivo inhibition would be similar. Recent studies have suggested that signaling through VEGFR-1 is directly or indirectly involved in tumor angiogenesis in several types of cancer (26–30). In addition, it has been reported that signaling through VEGFR-3 plays an important role in lymphangiogenesis and may be involved in lymphatic metastasis (31, 32).

Enzyme and receptor phosphorylation data have shown that KRN951 is selective in its VEGFR tyrosine kinase activity, a feature that distinguishes it from other dual-targeted or multitargeted small-molecule inhibitors, such as AZD2171 (targeting both VEGFR and c-Kit), SU11248 (targeting VEGFR, PDGFR, c-Kit, and Flt3), or BAY 43-9006 (targeting VEGFR, PDGFR, c-Kit, Flt3, and Raf). Although the inhibition of cancer-associated kinases by these multitargeted agents could provide additional therapeutic benefit in the treatment of certain types of tumors, it could lead to unwanted adverse side effects in patients through the inhibition of the physiologic function of the target kinase. For example, c-Kit plays an important role in the growth of gastrointestinal stromal tumors (33) and acute myeloid leukemia (34), but it is also critical in the development of the intestinal cells of Cajal, which show pacemaker activity (35, 36). It is therefore possible that c-Kit inhibition would cause gastrointestinal malfunctions such as diarrhea. Thus, selective VEGFR inhibitors are welcome from a safety viewpoint, particularly in cases where the tumors are less likely to depend on alternative kinases.

Progress in molecular-targeted cancer therapeutics highlights the need for suitable biomarkers to evaluate the efficacy of such treatments. Until recently, the most widely used method to analyze angiogenesis inhibitors was the qualification of intratumoral microvessel density in tumor biopsies. As a repeatable, noninvasive technique, DCE-MRI has been successfully used to monitor the pharmacokinetics and pharmacodynamics of such agents in vivo. For instance, oxygen permeation and drug delivery. The "vascular normalization" hypothesis predicts that a VEGF-R2 blockade would result in an increase in pericyte coverage of tumor vessels (41). Indeed, KRN951 increased the proportion of endothelial cells associated with pericytes, favoring this hypothesis. Of course, it could be assumed that this increase is still due to the preferential pruning of pericyte-poor vessels (42, 43). However, if the vascular normalization theory were validated, antiangiogenic therapy could be administered together with cytotoxic drugs, rendering it more effective in combating tumors. Whatever the exact mechanism of action of such a combined therapy, a recent study with bevacizumab provides definitive proof of the efficacy of angiogenesis inhibitors when combined with a chemotherapeutic regimen in the clinic (44). The enlargement of tumor vessel diameter and the increase in pericyte coverage after KRN951 treatment can be explained by this theory, suggesting a possible mode of action for KRN951 when associated with another antitumor agent.

It is conceivable that the knowledge of the target serum concentration, particularly of molecular-targeted drugs, would enable the design of appropriate regimens to ensure constant target suppression and the selection of individual dose optimizations in a clinical setting. The pharmacokinetic and pharmacodynamics analysis in the current study revealed that simulated KRN951 concentrations of ~70 ng/mL (~140 nmol/L) are sufficient to inhibit tumor growth in rats after oral administration. This estimate is much higher than the in vitro concentrations required to inhibit VEGF signaling and is likely due to plasma protein binding of KRN951, because our preliminary data indicate that the plasma protein binding ratio is >99% in rats. Follow-up studies are being conducted to define the pharmacokinetic variables that drive efficacy.

In this article, we describe the characterization of KRN951, a p.o. bioavailable angiogenesis inhibitor targeting VEGFR tyrosine kinases with potent antitumor efficacy. We also show that DCE-MRI is useful in detecting the in vivo antiangiogenic efficacy of KRN951 at an early stage of treatment. Furthermore, we show pharmacokinetic profiles that estimate the KRN951 serum concentration necessary for its in vivo antitumor efficacy. These results provide a basic rationale for further investigation of KRN951 as an antitumor agent in clinical settings. KRN951 is currently under evaluation in a phase I clinical trial.

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KRN951, a Highly Potent Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases, Has Antitumor Activities and Affects Functional Vascular Properties

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