The Thymidine Phosphorylase Inhibitor 5'-O-Tritylinosine (KIN59) Is an Antiangiogenic Multitarget Fibroblast Growth Factor-2 Antagonist

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Abstract

5'-O-Tritylinosine (KIN59) is an allosteric inhibitor of the angiogenic enzyme thymidine phosphorylase. Previous observations showed the capacity of KIN59 to abrogate thymidine phosphorylase–induced as well as developmental angiogenesis in the chicken chorioallantoic membrane (CAM) assay. Here, we show that KIN59 also inhibits the angiogenic response triggered by fibroblast growth factor-2 (FGF2) but not by VEGF in the CAM assay. Immunohistochemical and reverse transcriptase PCR analyses revealed that the expression of laminin, the major proteoglycan of the basement membrane of blood vessels, is downregulated by KIN59 administration in control as well as in thymidine phosphorylase– or FGF2-treated CAMs, but not in CAMs treated with VEGF. Also, KIN59 abrogated FGF2-induced endothelial cell proliferation, FGF receptor activation, and Akt signaling in vitro with no effect on VEGF-stimulated biologic responses. Accordingly, KIN59 inhibited the binding of FGF2 to FGF receptor-1 (FGFR1), thus preventing the formation of productive heparan sulphate proteoglycan/FGF2/FGFR1 ternary complexes, without affecting heparin interaction. In keeping with these observations, systemic administration of KIN59 inhibited the growth and neovascularization of subcutaneous tumors induced by FGF2-transformed endothelial cells injected in immunodeficient nude mice. Taken together, the data indicate that the thymidine phosphorylase inhibitor KIN59 is endowed with a significant FGF2 antagonist activity, thus representing a promising lead compound for the design of multtargeted antiangiogenic cancer drugs. Mol Cancer Ther; 11(4); 817–29. ©2012 AACR.

Introduction

Angiogenesis is the process by which new blood capillaries are generated from preexisting blood vessels. It is an essential and well-regulated process during embryogenesis, wound healing, and in the female reproductive cycle. However, angiogenesis is also associated with numerous pathologic processes (1–3) and is considered one of the hallmarks of cancer, being indispensable for tumor growth and metastasis (4). Therefore, the tumor vasculature is an attractive target for anticancer therapy.

Blood vessel formation is a multistep process, which involves (i) the degradation of the basement membrane and extracellular matrix (ECM) surrounding the existing vessel, (ii) migration, (iii) proliferation of the endothelial cells into the perivascular space, (iv) the formation of new vascular loops, with (v) the development of tight junctions, and (vi) the deposition of a new basement membrane and ECM. These different steps are regulated by a multitude of inhibitory and stimulatory signals, including growth factors, chemokines, adhesion molecules, and enzymes (2, 3). Among them, the VEGF family and cognate tyrosine kinase receptors VEGFR1–3 have represented a central focus in tumor angiogenesis research (5, 6), and the VEGF/VEGFR system has been extensively investigated as a target for antineoplastic therapy (7). In 2004, the anti-VEGF monoclonal antibody bevacizumab was approved as first-line therapy in combination with standard 5-fluorouracil–based chemotherapy in patients with advanced colorectal cancer. Since then, agents that target the activity of VEGFRs, such as the tyrosine kinase inhibitors sunitinib and sorafenib, have been approved for use in selected cancers (8).

Even though VEGF plays a central role in switching on a proangiogenic phenotype in most tumors, neoplastic, stromal, and infiltrating cells may produce a plethora of
different proangiogenic factors during tumor progression, recurrences, and metastases (9). Thus, VEGF/VEGFR antagonists may not be effective in the treatment of all cancers at all stages. Moreover, when the activity of VEGF is suppressed for a long period of time, resistance to VEGF/VEGFR2 blockade may occur (10, 11) because of the expression of other angiogenic factors, including fibroblast growth factor-2 (FGF2) and thymidine phosphorylase (2, 9, 12). These observations call for the development of novel antiangiogenic compounds, possibly characterized by a multitargeted mechanism of action.

FGF2 is one of the best characterized members of a large superfamily of structurally related cytokines that have potent mitogenic and angiogenic properties (13). Their biologic activities depend on the binding to high-affinity tyrosine kinase FGF receptors (FGFR) and low-affinity heparan sulphate proteoglycans (HSPG). The latter interaction prevents the proteolytic degradation of FGF2 in the extracellular environment and induces growth factor oligomerization, which facilitates FGFR dimerization and subsequent transactivation (13). As such, various compounds, such as soluble heparin, polysulfonated compounds, and FGF2-binding peptides, which abrogate the interaction of FGF2 with HSPGs and/or FGFRs, have been shown to inhibit the biologic activity of FGF2 and to possess antitumor activity in vivo (14–18).

Thymidine phosphorylase is an intracellular enzyme that catalyzes the conversion of thymidine and inorganic phosphate into thymine and 2-deoxy-D-ribose-1-phosphate. Thymidine phosphorylase is upregulated in several tumors, and high thymidine phosphorylase expression is associated with increased angiogenesis, tumor cell metastasis, and poor prognosis (19). The enzymatic activity of thymidine phosphorylase, and in particular, the dephosphorylated reaction product 2-deoxy-D-ribose, was found to be essential for its angiogenic effect (20, 21). In contrast to FGF2 or VEGF, no receptor for thymidine phosphorylase (or 2-deoxy-D-ribose) has been identified. Moreover, thymidine phosphorylase is not a growth factor. It is hypothesized that 2-deoxy-D-ribose, which can diffuse out of the cell, directly activates integrins αvβ3 and αvβ5, and focal adhesion kinase on endothelial cells, leading to cell migration (22).

We previously showed that the nucleoside derivative 5-O-trityl-2-deoxy-d-ribose (Kin59) inhibits the catalytic activity of thymidine phosphorylase (23). Accordingly, Kin59 completely prevented thymidine phosphorylase–induced angiogenesis in the chicken chorioallantoic membrane (CAM) assay. Moreover, the compound was far more potent than other thymidine phosphorylase inhibitors and also inhibited basal, developmental vascularization of the CAM (i.e., in the absence of exogenously added angiogenic factors; refs. 23–25). This suggests that Kin59 might also target angiogenic molecules other than thymidine phosphorylase. Because VEGF and FGF2 have been shown to be expressed in the developing CAM (26–28), we studied the effect of Kin59 on the angiogenic activity of these growth factors.

Our data indicate that, in addition to thymidine phosphorylase, Kin59 inhibits angiogenesis stimulated by FGF2, without affecting VEGF activity. The FGF2 antagonist activity of Kin59 results from its capacity to interfere with the formation of a productive HSPG/FGF2/FGFR ternary complex, required for receptor activation and biologic activity. In addition, the compound inhibited the growth of FGF2-overexpressing vascular tumors in mice. To our knowledge, Kin59 represents the first compound that combines thymidine phosphorylase and FGF2 antagonist activity. Thus, Kin59 might represent a promising lead compound for the design of a new class of multi-targeted antiangiogenic cancer drugs.

Materials and Methods

Compounds

Kin59 (Fig. 1A) was synthesized as described previously (23, 29).

Cell cultures

Fetal bovine aortic endothelial GM7373 cells (30) were obtained from the National Institute of General Medical Sciences (Bethesda, MD) and Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ) in 1990. No authentication was done since then. These cells were used to generate FGFR1-overexpressing cells (GM7373-FGFR1; ref. 14) and VEGFR2-overexpressing (GM7373-VEGFR2; ref. 31) cells in our laboratory. BALB/c mouse aortic endothelial 22106 cells (MAEC) and brain microvascular endothelial 10027 cells (MBEC) were obtained from R. Auerbach (University of Wisconsin, Madison, WI) in 1995 and were characterized by us (expression of endothelial markers, including oxLDL uptake, CD31, and KDR expression in 1997 as described; ref. 32). No further authentication was done since then. MAECs were used to generate FGF2-overexpressing FGF2-T-MAE cells in our laboratory. Wild-type Chinese hamster ovary (CHO)-K1 cells and A745 CHO cell mutants that were used in our laboratory to generate FGFR1-overexpressing CHO A745 flg-1A cells (33) were provided by J.D. Esko (University of California, La Jolla, CA) in 2000. No authentication was done since then except to confirm routinely that CHO A745 cell mutants do not express HSPGs. GM7373 cells and MBECs were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 10% FBS (Biochrom AG) and 10 mmol/L HEPES (Invitrogen). CHO-K1 cells and CHO A745 flg-1A were grown in Ham’s F-12 medium supplemented with 10% FBS. All cells were grown at 37°C in a humidified incubator with a gas phase of 5% CO2.

FGF2-T-MAE cells, which express high levels of the M, 18,000, M, 22,000, and M, 24,000 molecular weight isoforms of FGF2 (34), were transfected with the firefly luciferase–expressing vector pGL4.50 (Promega), using Nanojuice (Merck) according to the manufacturer’s instructions. Single-cell clones, stably expressing luciferase,
were established, and the brightest clone (F2T-luc2.9) was selected for further experiments. F2T-luc2.9 cells were maintained in DMEM, supplemented with 10 mmol/L HEPES, 10% FBS, 500 µg/mL geneticin (Invitrogen), and 500 µg/mL hygromycin (Invitrogen). These cells showed comparable growth, angiogenic and tumor-inducing properties as the parent cells, but were not further characterized.

Cell proliferation assays

GM7373 cells were seeded in 24-well plates at 60,000 cells per cm². After overnight incubation at 37°C, the cells

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were incubated for 24 hours in fresh medium containing 0.4% FBS, 30 ng/mL FGF2 or VEGF, and different concentrations of KIN59. At the end of the incubation period, cells were trypsinized and counted. In a second set of experiments, cells were treated with 30 ng/mL of FGF2 in the presence of KIN59. After 24 hours, cells were washed and incubated with fresh medium containing 10% FBS for another 24 hours. Next, cells were trypsinized and counted.

MBECs were seeded in 24-well plates at 25,000 cells per cm². After overnight incubation at 37°C, the cells were incubated in fresh medium containing 0.4% FBS, 30 ng/mL FGF2 or VEGF, and different concentrations of KIN59. After 48 hours, cells were trypsinized and counted.

F2T-luc2.9 cells were seeded in 48-well plates at 10,000 cells per cm². After 24 hours, the medium was replaced, and increasing concentrations of KIN59 were added. The cell cultures were incubated for 3 days, trypsinized, and counted in a Coulter counter analyzer (Analis).

**FGF2-mediated cell–cell adhesion assay**

This was conducted as described previously (35). Briefly, wild-type CHO-K1 cells were seeded in 24-well plates at 150,000 cells per cm². After 24 hours, cell monolayers were washed with PBS and incubated with 3% glutaraldehyde in PBS for 2 hours at 4°C. Fixation was stopped with 0.1 mol/L glycine, and cells were washed extensively with PBS. Then, CHO A745 flg-1A cells (50,000 cells per cm²) were added to CHO-K1 monolayers in serum-free medium plus 10 mmol/mL EDTA with or without 30 ng/mL of FGF2 in the absence or presence of increasing concentrations of KIN59. After 2 hours of incubation at 37°C, unattached cells were removed by washing twice with PBS, and CHO A745 flg-1A cells bound to the CHO-K1 monolayer were counted under an inverted microscope at ×125 magnification. Data are expressed as the mean of the cell counts of 3 microscopic fields chosen at random. All experiments were carried out in triplicate and repeated twice with similar results.

**BIAcore-binding assay**

The ability of KIN59 to inhibit the binding of FGF2 to immobilized heparin or to FGFR1 was investigated using surface plasmon resonance (SPR) technology (BIAcore X apparatus; GE Healthcare).

Heparin was immobilized to a BIAcore sensor chip as described previously (36). Briefly, a CM3 sensor chip previously activated with 50 μL of a mixture containing 0.2 mol/L 1-ethyl-3-(3-diaminopropyl)-carbodiimide hydrochloride (EDC) and 0.05 mol/L N-hydroxysuccinimide (NHS) was coated with streptavidin. Heparin was biotinylated at its reducing end and immobilized onto the streptavidin-coated sensor chip. These experimental conditions allowed the immobilization of 80 resonance units (RU) equal to 5.8 fmol/mm² of heparin. A sensor chip coated with streptavidin alone was used as a reference and for blank subtraction.

Recombinant human sFGFR1(IIIc)/Fc chimera (5 μg/mL in 10 mmol/L sodium acetate, pH 3.0) was allowed to react with a flow cell of a CM5 sensor chip. These experimental conditions allowed the immobilization of 6.183 RU, equal to 0.34 pmol/mm² of sFGFR1(IIIc)/Fc chimera (18). Similar results were obtained for immobilization of the irrelevant Noggin-Fc protein chimera, here used as a negative control and for blank subtraction. In both experimental models, FGF2 (50 nmol/L) was injected in the presence of increasing concentrations of the compound under test in HBS-EP buffer (0.01 mol/L HEPES, pH 7.4 plus 0.005% surfactant P20, 0.15 mol/L NaCl, 3 mmol/L EDTA) for 4 minutes to allow the association of FGF2 with the immobilized ligand. The response (in RU) was recorded as a function of time.

**Western blot analysis**

Serum-starved FGFR1-overexpressing GM7373 cells (GM7373-FGFR1 cells; ref. 14) and VEGFR2-overexpressing GM7373 cells (GM7373-VEGFR2 cells; ref. 31) were incubated with 60 μmol/L KIN59 or vehicle for 30 minutes, after which 10 ng/mL FGF2 or 30 ng/mL VEGF was added. After 10 minutes of stimulation, cells were lysed and 50 μg aliquots of the cell extracts were analyzed by SDS-PAGE followed by Western blotting with anti-P-FGFR1 antibody (Santa Cruz Biotechnology), anti-p-Akt antibody (Cell Signaling Technology), and anti-p-VEGFR2 antibody (R&D Systems). In all the experiments, uniform loading of the gels was confirmed using anti-FAK antibody (Santa Cruz Biotechnology).

**CAM gelatin sponge assay**

The in vivo CAM gelatin sponge model was conducted as described by Ribatti and colleagues (37) with slight modifications. Fertilized chicken eggs were incubated for 3 days at 37°C at constant humidity. After removal of 3 mL of albumin to detach the developing CAM from the shell, a square window was opened in the eggshell, exposing the CAM. The window was sealed with cellophane tape, and the eggs were returned to the incubator. At day 9 of embryonic development, 2-mm³ sterilized gelatin sponges (Gelfoam, Upjohn) containing the test compounds were placed on top of the CAM. These sponges contained either thymidine phosphorylase (5 μL containing 5 U; Sigma), FGF2 (500 ng in 5 μL; Sigma), or VEGF165 (500 ng in 5 μL; R&D systems) alone or with KIN59 (10 μL containing 125 nmol in 40% cremophor-PBS) or 40% cremophor-PBS (10 μL). Forty-eight hours later, the sponges were processed for zymography, reverse transcriptase PCR (RT-PCR), histologic, or immunohistochemical analysis. Forty percent of cremophor [cremophor EL; Sigma; instead of dimethyl sulfoxide (DMSO)] was used to dissolve KIN59, because slight toxicity was observed in sponges treated with DMSO. Cremophor is a derivative of castor oil and ethylene oxide.
Histologic and immunohistologic analysis of the sponges
CAMs were fixed in ovo in 4% paraformaldehyde in PBS for 1 hour, dehydrated in graded embedded in paraffin, and serially sectioned at 7 μm (4 μm for immunohistochemistry) along a plane parallel to their free surface. For histology, sections were stained with hematoxylin and eosin (H&E). To determine the number of blood vessels, pictures of the CAM tissue around the gelatin sponges at a ×100 magnification were taken (with the Zeiss Imager Z1 microscope, Zeiss), and the blood vessels on these pictures were counted in a double-blind fashion. The mean blood vessel counts ± SD were determined for each analysis. For statistical analyses, the P values were determined using the Student t test, and P values < 0.05 were considered significant.

After dehydration of the tissue sections, the slides were washed in PBS for 15 minutes and incubated in 1% trypsin (in 0.1% CaCl2) at 37°C for 30 minutes for antigen retrieval. Next, the tissue sections were incubated overnight with mouse anti-laminin antibody (L8271; Sigma) in PBS containing 1% bovine serum albumin to prevent nonspecific binding. After 3 washes of 5 minutes in PBS, the slides were sequentially incubated with a biotin-labeled anti-mouse secondary antibody (Vector) for 60 minutes and with streptavidin-peroxidase conjugate (Vector) for 30 minutes. Sections were washed in PBS and counterstained with Mayer’s hematoxylin for 10 minutes and mounted in glycerol (glycergel; Dako). In the negative controls, no primary antibodies were applied.

To quantify the staining, pictures of 0.45 mm² were taken at ×100 magnification with a Zeiss Imager Z1 microscope. For each condition, at least 9 samples (3 sponges from 3 independent experiments) were taken, and 2 sections per sample were stained and photographed. The pictures were always taken of a CAM area adjacent to the sponge. Next, the stained area was calculated using Axiovision software. Staining was rated as a percentage of the photographed area. The mean values ± SD were calculated per sample and per group of samples. For statistical analyses, the Student t test was used, and P values < 0.05 were considered significant.

Real-time RT-PCR
The CAM gelatin sponges were incubated in 700 μL RLT buffer (Qiagen) and homogenized as described earlier. After centrifugation for 20 minutes at 4°C and 13,000 rpm, the cleared supernatant was used for total RNA isolation with the RNeasy Mini Kit (Qiagen). To eliminate potential genomic DNA contamination, the samples were treated with DNase I (Roche). Two hundred nanograms of total RNA was reverse-transcribed to cDNA by means of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and Oligo(dT)18 Primer (Invitrogen) according to the manufacturer’s instructions.
Quantitative real-time PCR was carried out with an ABI Prism 7000 sequence detection system (Applied Biosystems). Primers were designed according to the chicken sequences of the A1 chain of laminin (5'-GCCCTAGTCGATGATCC-3' and 5'-GCCGACGAGAGGATGTC-3') and the housekeeping genes hypoxanthine phosphoribosyltransferase (HPRT, 5'-TGGCGATGAAACAGTG-3' and 5'-GCTACATGTTGCTTCC-3'), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 5'-GGAGTCAA-CGGATTGCCC-3' and 5'-TCTCCAGAGGACGCC-3'), and β-actin (5'-ATGGCTCCGTATGTGCAA-3' and 5'-TGTC-TTCTGGCCCATACCA-3').
All samples were analyzed in triplicate in a final volume of 25 μL containing SYBR green PCR Master Mix 30 (Eurogentec) containing 300 nmol/L of each primer (Invitrogen) and 40 ng of cDNA template. The thermal cycle parameters used were 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. After 40 cycles, samples were run using the dissociation protocol to detect any nonspecific amplification. The results were evaluated by use of SDS software (Applied Biosystems). For each sample, the relative amount of laminin mRNA was determined and normalized to 3 housekeeping genes (GAPDH, HPRT, and β-actin) using the ΔΔCt method (38). The calibrator used was a control gelatin sponge incubated with cremophor. For statistical analyses, the Student t test was used, and P < 0.05 was considered significant.

Tumor growth in mice
Eight-week-old female, athymic, nude nu/nu mice, weighing about 25 g, were inoculated subcutaneously with 200 μL of serum-free DMEM containing 2 × 10⁶ F2T-luc2.9 cells. KIN59 treatment was started after 24 hours and continued till the end of the experiment. The compound was administered subcutaneously at 15 mg/kg, twice daily (once daily during the weekend) at a site distant from the tumor (inoculation) site. The mice were imaged at regular time intervals to assess the growth of the luciferase-positive tumor cells. Before imaging, the mice were injected i.p. with 200 μL of a 40 mg/mL luciferin solution in PBS. Images were captured by the IVIS Spectrum Imaging System (Caliper Life Sciences) and analyzed using the LivingImage Software (Caliper Life Sciences). Tumor size was monitored by means of a caliper, and the tumor volume was calculated with the following formula: tumor volume (mm³) = 0.5 × a × b², where a is the longest diameter and b is the shortest diameter. At the end of the experiment, the tumors were excised, weighed, and processed for histologic analysis. For statistical analyses, the Student t test was used, and P < 0.05 was considered significant. All studies were done in compliance with the ethical guidelines for animal welfare of the KU Leuven, Leuven, Belgium.
Histologic and immunohistochemical analysis of the tumors

Tumors and organs were fixed in 4% paraformaldehyde for 24 hours, rehydrated, and embedded in paraffin. Deparaffinized sections were subsequently stained with H&E and examined microscopically.

For immunohistochemical analysis of FL2-luc2.9 tumors, deparaffinized sections were rehydrated, microwaved in 10 mmol/L citrate buffer (pH 6.0) to retrieve the antigens, and incubated in 0.5% H2O2 for 30 minutes to quench endogenous peroxidase activity. Next, the slides were incubated for 20 minutes with 3% goat serum to block nonspecific binding sites, washed 3 times in PBS, and incubated with rabbit anti-CD31 antibody (Abcam) or anti-laminin antibody (Abcam) for 1 hour. After washing with PBS, the slides were incubated with peroxidase-labeled goat anti-rabbit secondary antibody (Dako) for 30 minutes and subsequently visualized using the Dako Envision System (Dako). Finally, the sections were counterstained with hematoxylin and mounted.

To analyze the tumor vasculature, 2 representative pictures were taken from each tumor at ×40 magnification and the areas of the highly vascular, vascular, and avascular zone were calculated with the item FEI Nikon software (Nikon). To quantify laminin staining, 2 sections per sample were stained and for each section, 2 double-blind pictures were photographed. Next, the stained brown immunopositive area was calculated using Axiovision software (Zeiss). Staining was rated as a percentage of the photographed area. For statistical analyses, the Student t test was used, and P < 0.05 was considered significant.

Results

KIN59 inhibits FGF2-induced but not VEGF-mediated neovascularization in the CAM assay

KIN59 inhibits basal and thymidine phosphorylase–induced angiogenesis in the CAM assay (23). The modest anti-thymidine phosphorylase activity of KIN59 could not explain its potent antiangiogenic activity. Therefore, we investigated the effect of KIN59 on angiogenesis induced by FGF2 and VEGF, both expressed in the developing CAM (26–28). The CAM gelatin sponge assay was applied (37) because this technique allows histologic and immunohistochemical analysis of newly formed blood capillaries and the surrounding ECM.

First, increasing amounts of KIN59 (10–250 nmol) were applied onto the CAM in the absence of any exogenously added angiogenic factor. The maximal antiangiogenic effect was obtained with 125 nmols per sponge (data not shown), and this concentration was used in further experiments. Next, the effect of KIN59 on thymidine phosphorylase-, FGF2- and VEGF-induced angiogenesis was evaluated. As shown in Fig. 1, thymidine phosphorylase, FGF2, and VEGF induced, respectively, a 2.4-, 2.5-, and 2.1-fold increase in CAM vascularization when compared with PBS-treated CAMs (P < 0.001). KIN59 (125 nmols/sponge) strongly inhibited neovascularization induced by thymidine phosphorylase or FGF2 (86% and 84% inhibition, respectively; P < 0.001). In contrast, the angiogenic activity of VEGF was not affected, thus indicating that this growth factor was able to overcome also the inhibitory effect exerted by KIN59 on basal angiogenesis (Fig. 1B and C). Addition of vehicle (40% cremophor in PBS) had no influence on basal angiogenesis or on the angiogenic properties of the stimulatory factors.

Blood microvessels consist of endothelial cells surrounded by a basement membrane, a 3-dimensional network of interconnecting glycoproteins and collagens essential for proper endothelial cell function (39, 40). Because laminin is the major endothelial cell basement membrane glycoprotein (41), we investigated whether the antiangiogenic effect of KIN59 in the CAM assay was correlated to changes in laminin expression. Immunohistochemistry of CAM implants showed that laminin immunoreactivity, present in the basement membrane of blood vessels, epithelium and ECM of untreated samples, is abolished by KIN59 treatment (Fig. 2A and B). KIN59 also significantly reduced the levels of laminin in sponges treated with thymidine phosphorylase or FGF2 but not in sponges treated with VEGF (Fig. 2A and B). However, KIN59 did not affect type I collagen, type IV collagen, or fibronectin immunoreactivity both in basal and angiogenic factor–stimulated CAMs, thus confirming the specificity of the effect (data not shown). On this basis, we investigated the effect of KIN59 on laminin mRNA expression in CAM implants using quantitative real-time RT-PCR analysis. For each sample, the relative levels of laminin mRNA were determined and normalized to 3 housekeeping genes (GAPDH, HPRT, and β-actin). KIN59 caused a 10-fold reduction in laminin mRNA levels in CAMs treated with thymidine phosphorylase or FGF2, but it did not affect laminin expression in VEGF-containing implants (Fig. 2C). At variance, gelatin zymography on CAM extracts did not show any effect of KIN59 on the activity of the laminin degrading matrix metalloproteinase (MMP)-2 (42) both in basal and angiogenic factor–stimulated CAMs (data not shown), whereas MMP-9 could not be detected in the CAM extracts (see also refs. 42, 43).

Taken together, these results indicate that KIN59 selectively suppresses developmentally regulated and thymidine phosphorylase- or FGF2-triggered blood vessel formation and laminin deposition in the chick embryo CAM with no effect on VEGF-induced angiogenesis.

KIN59 inhibits FGF2- but not VEGF-stimulated endothelial cell growth

FGF2 and VEGF, but not thymidine phosphorylase, are potent endothelial cell mitogens. On this basis, we evaluated the effect of KIN59 on endothelial cell proliferation induced by FGF2 or VEGF. As shown in Fig. 3A, KIN59 inhibited FGF2-induced proliferation of bovine macrovascular endothelial cells (37) because this technique allows histologic and immunohistochemical analysis of newly formed blood capillaries and the surrounding ECM.

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FGF2 and VEGF, but not thymidine phosphorylase, are potent endothelial cell mitogens. On this basis, we evaluated the effect of KIN59 on endothelial cell proliferation induced by FGF2 or VEGF. As shown in Fig. 3A, KIN59 inhibited FGF2-induced proliferation of bovine macrovascular endothelial CM7373 cells in a dose-dependent manner with a 50% inhibitory concentration (IC50) of
5.8 μmol/L, a potency 10 times higher than that shown to inhibit the proliferation induced by 10% FBS (IC₅₀ = 63 μmol/L). In agreement with the CAM data, KIN59 (at 30 μmol/L) did not affect the growth-promoting activity of VEGF in GM7373 cells (Fig. 3B). Similar results were obtained when KIN59 was tested on murine microvascular MBECs (data not shown). Interestingly, the inhibitory effect exerted by KIN59 on FGF2-induced GM7373 cell proliferation seems to be cytostatic (i.e., fully reversible after fresh medium replacement after 24 hours treatment with the compound) for KIN59 concentrations up to 30 μmol/L and cytotoxic only at higher concentrations (data not shown).

Accordingly, KIN59 inhibited FGFR1 phosphorylation and Akt activation triggered by FGF2 in FGFR1-overexpressing GM7373 cells (Fig. 3C). KIN59 significantly decreased the mRNA and protein level of laminin in sponges treated with PBS, thymidine phosphorylase, and FGF2, whereas it had no effect on the laminin expression in VEGF-treated sponges.

KIN59 inhibits the interaction of FGF2 with FGFR1

FGF2 exerts its biologic activity by leading to the formation of productive HSPG/FGF/FGFR ternary complexes in target cells (13). To investigate whether KIN59 may inhibit the angiogenic activity of FGF2 by preventing the formation of this complex, a FGF2-dependent cell–cell adhesion model was used in which the growth factor mediates the interaction in trans of HSPG-deficient CHO cells overexpressing FGFR1 to a monolayer of HSPG-bearing CHO-K1 cells (35). As shown in Fig. 4A, KIN59 inhibits the interaction of FGF2 with its high-affinity tyrosine kinase receptor. As a consequence, formation of the ternary HSPG/FGF2/FGFR1 complex is hampered, resulting in the inhibition of FGF2 biologic activity.

KIN59 inhibits the growth of FGF2-overexpressing tumors

To investigate the ability of KIN59 to affect FGF2-dependent tumor growth in vivo, we took advantage of...
a highly tumorigenic FGF2-overexpressing mouse aortic endothelial cell line (FGF2-T-MAE cells) generated in our laboratory (34). These cells are characterized by a sustained rate of growth in vitro and by the capacity to generate opportunistic vascular lesions in nude mice as a consequence of an autocrine loop of stimulation triggered by the release of the overexpressed growth factor (44). Accordingly, different FGF2 antagonists inhibit the growth of FGF2-T-MAE cells in vitro and in vivo (14, 15, 17, 18). On this basis, as a tool for further in vivo experimentation, FGF2-T-MAE cells were stably transfected with firefly luciferase, thus generating F2T-luc2.9 cells.

In vitro, KIN59 inhibited the proliferation of F2T-luc2.9 cells with an IC_{50} of 44 \pm 9 \mu mol/L and 65 \pm 4 \mu mol/L when cells were grown in 0.4% or 10% FBS, respectively. Next, F2T-luc2.9 cells were injected subcutaneously into the flanks of female nu/nu mice and monitored for 3 weeks using bioluminescent imaging and caliper measurements. Subcutaneous treatment (at a site distant from the inoculation site) with KIN59 was started 24 hours after injection of the cells. Because of the low solubility of KIN59, the compound was administered in a mixture of DMSO/cremophor in PBS twice daily at 15 mg/kg. Control mice received equal volumes of the vehicle. As shown in Fig. 5A and B, KIN59 caused a significant inhibition in the rate of tumor growth when compared with vehicle, as assessed both by bioluminescence and tumor volume measurements. This was confirmed by measurement of tumor weight at sacrifice (0.41 \pm 0.07 g vs. 0.23 \pm 0.03 g for vehicle- and KIN59-treated animals, respectively; \( P < 0.05 \); Fig. 5C). No reduction in body weight was observed during the course of treatment. Also, histologic analysis of the different organs did not reveal any signs of toxicity (not shown).

Histologically, F2T-luc2.9 tumors consisted of a highly vascularized rim of spindle-shaped cells surrounding an area with lower blood vessel density; the tumor core instead was poorly vascularized but consisted of viable tumor cells without any evidence of necrosis or apoptosis (Fig. 6A). KIN59-treated tumors, even though smaller in size, showed a similar tissue organization. However, immunohistochemical analysis for CD31 (Fig. 6B) showed that the avascular area of the lesions was more extended when compared with vehicle-treated tumors (62% \pm 6% vs. 24% \pm 5% of the tumor section area for KIN59- and vehicle-treated animals, respectively; \( P < 0.05 \)). Moreover, in control sections, laminin was highly expressed in the ECM, predominantly at the tumor edge and surrounding the blood vessels (Fig. 6C). However, in KIN59-treated tumors, laminin was expressed at significantly reduced levels.
levels (10.7% ± 1.6% of the tumor section area for control vs. 1.1% ± 0.4% for KIN59-treated tumors, \( P < 0.01; \) Fig. 6D) and was only detected at a small rim at the border of the tumors and not in the basement membrane of the endothelial cells (Fig. 6C). Together, these data suggest that inhibition of tumor vascularization and laminin deposition contribute to the antitumor activity of KIN59.

Discussion

The nucleoside derivative KIN59 was first described as an inhibitor of the angiogenic enzyme thymidine phosphorylase (23). Although the anti–thymidine phosphorylase activity of KIN59 was rather modest, the compound completely blocked thymidine phosphorylase–induced as well as endogenous angiogenesis in the chick embryo CAM assay, suggesting that this compound might also affect the activity of other proangiogenic factors expressed in the developing CAM, including FGF2 (26, 27). Indeed, previous observations had shown the capacity of a neutralizing anti–FGF2 antibody to fully block developmental vascularization of this embryonic adnexum (28).

In this study, we show that KIN59 fully inhibits the angiogenic activity exerted by FGF2 exogenously added to the chick embryo CAM via a gelatin sponge implant. Under the same experimental conditions, the compound had no effect on the neovascular response elicited by VEGF. In keeping with these observations, KIN59 also inhibited FGF2-induced proliferation of endothelial cells of different origin whereas it had no effect on VEGF-stimulated endothelial cell growth. Also, KIN95 suppressed FGFR1 phosphorylation and Akt activation triggered by FGF2, whereas the compound did not inhibit VEGFR2 activation and signaling triggered by VEGF. Thus, the FGF2 antagonist activity of KIN59 may explain its capacity to inhibit developmental vascularization of the chick embryo CAM.

The ability of KIN59 to antagonize FGF2 activity was rather surprising. KIN59 is an atypical inhibitor of thymidine phosphorylase (19), an intracellular enzyme for which no receptor has been identified. Unlike all previously described thymidine phosphorylase inhibitors, KIN59 does not interact with the thymidine- or phosphate-binding site of thymidine phosphorylase, indicating that the compound binds to an allosteric site in the enzyme. Computational studies on the thymidine phosphorylase/KIN59 complex identified a possible allosteric site involving amino acid residue Asp203 of human thymidine phosphorylase (45). At variance with thymidine phosphorylase, FGF2 is an extracellular growth factor whose biologic activity is mediated by the activation of tyrosine kinase FGFRs on the cell surface (13, 46). FGF2 also binds to HSPGs that act as low-affinity coreceptors, facilitating FGFR dimerization and signaling. Here, we show that KIN59 abolishes the formation of this productive HSPG/FGF2/FGFR1 ternary complex as a consequence of its ability to prevent FGF2/FGFR1 interaction, as shown by SPR analysis in which the compound inhibits the interaction of FGF2 with the extracellular domain of FGFR1 immobilized to the sensor chip. Under the same experimental conditions, the compound does not interfere with the binding of the growth factor to immobilized heparin, closely related in structure to heparan sulfate. Thus, KIN59 seems to differ from various negatively charged FGF2 inhibitors identified so far, including polysulfonated and polysulfated molecules, able to bind the basic domain of FGF2, thereby preventing its interaction with HSPGs (15). Indeed, in contrast to these compounds (14), KIN59 is uncharged and does not protect the growth factor from proteolytic digestion (data not shown).
Recently, we have shown that the pattern recognition receptor long pentraxin-3 (PTX3) and the PTX3-derived N-acetylated pentapeptide ARPCA (in single letter code) bind FGF2 with high affinity and specificity, thus preventing HSPG/FGF2/FGFR1 ternary complex formation and angiogenesis (18, 47). This effect seems to be because of the ability of these molecules to bind the receptor-binding region in the FGF2 protein, thereby hampering FGF2/FGFR1 interaction, without affecting heparin binding (18). However, KIN59 does not prevent the binding of PTX3 or ARPCA to FGF2, as assessed by SPR analysis, suggesting that KIN59 inhibits FGF2/FGFR1 binding by interacting with a new, as yet unidentified, site in the growth factor or its receptor (data not shown). Further experiments are required to fully characterize the molecular bases of the FGF2 antagonist activity of KIN59.

In agreement with the angiogenesis data, immunohistochemical analysis showed a significant reduction in the expression of laminin, a crucial component of the capillary basement membrane, in CAM sponges treated with PBS, thymidine phosphorylase, and FGF2, but not in sponges treated with VEGF. The basement membrane, consisting mainly of laminin and type IV collagen, provides structural stability to the vessels and supports key signals that regulate endothelial migration, proliferation, tube formation, and survival (39, 48). Remodeling of the basement membrane and ECM, through cycles of proteolytic degradation (e.g., by MMPs) and deposition of ECM components is essential for the angiogenic process. KIN59 did not change the expression or activity of MMP-2, whereas MMP-9 could not be detected in the CAM extracts. Thus, although it cannot be ruled out that other chick embryo proteases (43) are affected by KIN59, increased gelatinase activity does not seem to be responsible for the absence of laminin in KIN59-treated CAMs. Instead, RT-PCR analysis showed a 10-fold reduction in laminin mRNA, which indicates that KIN59 inhibits laminin biosynthesis by the CAM cells. In contrast, the compound did not change the expression of other ECM components, such as type IV collagen, type I collagen, and fibronectin (data not shown). Still, laminin deficiency alone may alter the firmness and architecture of the basement membrane, which may impact signal transduction by connecting proteins, such as integrins. Lack of the ability to provide survival signals may then lead to instability of the newly formed vessels, as observed in the presence of KIN59 (23, 40).

FGF2 has been implicated in tumor growth/vascularization and in tumor evasion from anti-VEGF therapies (reviewed in ref. 49). On this basis, to investigate the impact of KIN59 on FGF2-dependent tumor growth and vascularization, we used a firefly luciferase–transfected clone (F2T-luc2.9 cells) derived from the highly tumorigenic FGF2-overexpressing FGF2-T-MAE cells (34). These cells generate opportunistic vascular lesions when injected subcutaneously in nude mice as a consequence of an autocrine loop of stimulation triggered by the release of the overexpressed growth factor, thus representing a valid model to study FGF2-driven tumor growth and vascularization (44). As observed for other FGF2 antagonists (44, 47, 50), KIN59 inhibited the growth of FGF2-T-MAE cell–derived F2T-luc2.9 cells in vitro and significantly affected their growth in vivo. Also, KIN59-treated tumors showed a significant decrease in vascularity of the lesions and displayed strongly reduced laminin expression in the basement membrane and ECM, suggesting that inhibition of angiogenesis and modulation of the ECM may, at least in part, be responsible for the antitumor activity of KIN59.

Despite its interesting spectrum of activities, at least 2 aspects of KIN59 properties deserve further...
discussion: (i) the narrow therapeutic window of the compound due, at least in part, to its low solubility and (ii) its incapacity to affect VEGF-induced neovascularization.

Because of its low solubility, KIN59 could only be administered at 15 mg/kg, which is why the compound was injected twice daily (subcutaneously, at a site distant from the cell inoculation site). Low aqueous solubility is a common problem in the development of biologically active compounds. Fortunately, various approaches may be applied to increase the solubility of highly lipophilic compounds. In particular, we managed to increase the water solubility (and oral bioavailability) of poorly soluble amine-containing parent drugs up to 1,000-fold by using a dipeptidyl peptidase IV (DPPIV/CD26)-based prodrug approach (51, 52). A similar approach is in progress in our laboratory to generate more soluble KIN59 prodrugs.

Figure 6. Immunohistochemical analysis of KIN59-treated tumors. A, H&E staining shows the presence of 3 distinct areas in F2T-luc2.9-induced tumors: a highly vascularized (hv) outer rim, surrounding an area with lower blood vessel density (vascular area, v), and a largely avascular (av) inner core. Both control and KIN59-treated tumors show a comparable tissue organization. However, KIN59-treated tumors contain a significantly larger avascular core than the vehicle-treated tumors, as measured by CD31 staining of the blood vessels (B). Data are presented as mean ± SEM, n = 3, *P < 0.05 compared with control. Representative photographs of the different tumor areas stained with anti-CD31 (brown) are shown.

C and D, immunohistochemical staining for laminin in control and KIN59-treated tumors (C). In control tumors, laminin is detected in the ECM (top; predominantly at the tumor edge) and in the basement membrane of the endothelial cells (bottom, white arrowheads). In KIN59-treated tumors, laminin is only present in a small rim at the tumor border (top, arrows) and not surrounding the blood vessels (white arrowheads and bottom). D, the immunopositive area was quantified as described in Materials and Methods. KIN59 significantly decreased the expression of laminin. Values are expressed as mean ± SEM. n = 3, *P < 0.01.
The second point, the lack of VEGF activity of KIN59, may not necessarily represent a disadvantage. In fact, KIN59 may be administered together with an anti-VEGF drug or used to treat tumors that produce FGF2 and/or thymidine phosphorylase as their predominant angiogenic proteins. Moreover, clinical studies with drugs that target VEGF or VEGF-induced signaling, such as the VEGF-antibody bevacizumab (Avastin; Genentech/Roche) and the kinase inhibitors sorafenib and sunitinib showed that the benefits from these antiangiogenic therapies are at best temporary and usually followed by the development of tumor resistance (53). Tumor resistance may be caused by circumvention of the angiogenic blockade by activation and/or upregulation of alternative proangiogenic pathways, including thymidine phosphorylase and FGF2 (2, 9, 12). Thus, KIN59 may be used as a second-line approach after evasion from anti-VEGF/VEGFR therapies has occurred.

In conclusion, this study shows that KIN59 is a potent inhibitor of angiogenesis with antagonist activity against at least 2 angiogenic factors, namely, thymidine phosphorylase and FGF2. KIN59 also inhibits the deposition of the essential basement membrane component laminin and causes the degradation of newly formed blood vessels (23). As such, KIN59 might be a promising lead compound for the design of novel multitargeted anticancer drugs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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The Thymidine Phosphorylase Inhibitor 5’-O-Tritylinosine (KIN59) Is an Antiangiogenic Multitarget Fibroblast Growth Factor-2 Antagonist

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