Antagonism of Sphingosine-1-Phosphate Receptors by FTY720 Inhibits Angiogenesis and Tumor Vascularization

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Abstract

FTY720, a potent immunomodulator, becomes phosphorylated *in vivo* (FTY-P) and interacts with sphingosine-1-phosphate (S1P) receptors. Recent studies showed that FTY-P affects vascular endothelial growth factor (VEGF)–induced vascular permeability, an important aspect of angiogenesis. We show here that FTY720 has antiangiogenic activity, potently abrogating VEGF- and S1P-induced angiogenesis *in vivo* in growth factor implant and corneal models. FTY720 administration tended to inhibit primary and significantly inhibited metastatic tumor growth in a mouse model of melanoma growth. In combination with a VEGFR tyrosine kinase inhibitor PTK787/ZK222584, FTY720 showed some additional benefit. FTY720 markedly inhibited tumor-associated angiogenesis, and this was accompanied by decreased tumor cell proliferation and increased apoptosis. In transfected HEK293 cells, FTY-P internalized $S1P_1$ receptors, inhibited their recycling to the cell surface, and desensitized S1P receptor function. Both FTY720 and FTY-P apparently failed to impede VEGF-produced increases in mitogen-activated protein kinase activity in human umbilical vascular endothelial cells (HUVEC), and unlike its activity in causing S1PR internalization, FTY-P did not result in a decrease of surface VEGFR2 levels in HUVEC cells. Pretreatment with FTY720 or FTY-P prevented S1P-induced Ca^{2+} mobilization and migration in vascular endothelial cells. These data show that functional antagonism of vascular S1P receptors by FTY720 potently inhibits angiogenesis; therefore, this may provide a novel therapeutic approach for pathologic conditions with dysregulated angiogenesis. (Cancer Res 2006; 66(1): 221-31)

Introduction

Angiogenesis, the formation of new blood vessels from preexisting vessels, is a normal aspect of the physiologic remodeling processes that occurs in wound healing and during the female reproductive cycle. However, in pathologic situations, such as rheumatoid arthritis, diabetic retinopathy, and tumor development, abnormally enhanced neovascularization is a major contributory factor for disease progression (1, 2). The initiation of pathology-associated angiogenesis involves vascular permeability changes, driven by angiogenic factors, such as vascular endothelial growth factor (VEGF; ref. 3). This leads to fibrin deposition, plasmin activation, basement membrane degradation, and ultimately endothelial cell migration and proliferation, recruitment of mural cells, and vessel maturation (4).

Sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite secreted by platelets upon activation, is a potent proangiogenic molecule, which acts by binding various members of the G-protein–coupled receptor (GPCR) family of S1P receptors (S1P-R; refs. 5, 6). A novel immunosuppressant agent currently in clinical trials for renal transplant rejection (FTY720) and its metabolite of cellular kinase(s) FTY720 phosphate (FTY-P; ref. 7) bear structural similarity to sphingosine and S1P, respectively. FTY-P binds at low nanomolar concentrations to four of five S1P-Rs, $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ (8). Recently, we have shown that FTY-P can act in a similar manner to S1P, stimulating endothelial cell signaling, migration, survival, and differentiation (9). By recruiting adherens junction proteins to the endothelial cell-cell junctions (10), FTY720 has also been shown to antagonize VEGF-induced permeability of blood vessels (10, 11). Tumor-associated blood vessels are permeable and elicit tissue extracellular fluid extravasation; therefore, this prompted us to investigate whether FTY720 exerts any antiangiogenic and antitumor activity *in vivo* by affecting vessel permeability.

In this report, we show that FTY720 at clinically relevant doses, inhibits both S1P- and VEGF-induced angiogenesis, and impedes primary and metastatic tumor growth in a murine model of melanoma. Additionally, combination of FTY720 with the VEGFR tyrosine kinase inhibitor PTK787/ZK222584 (PTK/ZK) further reduces the growth of the tumors and metastases. These findings suggest that targeting S1P receptors may provide a novel therapeutic approach in cancer treatment.

Materials and Methods

Materials. S1P was purchased from BioMol Research Labs, Inc. (Plymouth Meeting, PA). FTY720 and all other related compounds mentioned herein were generously provided by the Novartis Transplantation Group (Basel, Switzerland) and prepared as described previously (9). Human umbilical vein endothelial cells (HUVEC), from Vec Technologies, Inc. (Rensselaer, NY), were maintained in MCDB 131 Complete media (Vec Technologies) and were used from passages 4 to 7.

Female C57/Bl6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or IFFA Credo (L'Arbresle, France). Female mice (MAG and NIH/Tif), weighing 18 to 20 g (6-8 weeks old), were obtained from the Novartis animal breeding facility. All animal experiments done in Switzerland were done in strict adherence to the Swiss law for animal protection,

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PTK787/ZK222584 is a co-development compound by Novartis AG and Schering AG.

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and experiments done in The United States were conducted in accordance with the Novartis Animal Care and Use Committee.

The S1P analogues FTY720, NVP-AAL149, and NVP-AAL151 and the VEGFR receptor tyrosine kinase inhibitor NVP-AAL993 were synthesized by Novartis AG (Basel, Switzerland). The VEGFR receptor tyrosine kinase inhibitor PTK/ZK was synthesized by Schering AG (Berlin, Germany) in collaboration with Novartis.

Fluorescence imaging plate reader Ca^{2+} mobilization assay. Fluorescence imaging plate reader (FLIPR) assay with HUVECs was carried out as described previously (9). Briefly, titrated compounds were preincubated with cells in a 96-well plate for 3 hours. Subsequently, the cell plates and ligand plates (containing 500 nmol/L S1P final concentration) were loaded into the FLIPR. The inhibition of S1P-induced calcium mobilization by FTY720, FTY-P, and NVP-AAL151 was plotted with EXCEL and SigmaPlot, and IC_{50} was determined for each compound.

Migration assay. The 4-hour migration assay was carried out using the BD Biocoat FluoroBlok System as described previously (9). HUVECs were preincubated for 30 minutes with compounds in MCDB 131 basal media (Vec Technologies), containing 0.1% bovine serum albumin (BSA, delipidized, BD Biosciences). S1P was diluted in the same media to a final concentration of 500 nmol/L and added to the bottoms of the assay plate wells. Migrated cells were stained with Calcein AM (Molecular Probes, Eugene, OR) and quantified with a CytoFluor II (PerSeptive Biosystems, Framingham, ME) fluorescent plate reader.

Mouse corneal micropocket assay. This method has been previously described in detail (12). Briefly, pellets containing the slow-release polymer Hydron and sucralfate with 180 ng $rHuVEGF_{165}$ were implanted into the cornea of female C57BL/6J mice. Daily oral treatment with FTY720 (0.3 or 3 mg/kg) or vehicle (10 mL/kg, 5% w/v glucose) was started 24 hours later. The eyes were routinely examined by slit-lamp biomicroscopy (Nikon FS-3V), and on day 6, mice were sacrificed, and the vascular response was quantified using a linear reticule through the slit lamp. Inhibition was determined by the formula $0.2\pi \times$ new blood vessel length $\times$ clock hours of neovessels. The circumferential zone was measured as clock hours with a 360-degree reticule (where 30 degrees of arc equals 1 clock hour). The data are reported as the % inhibition of blood vessel growth compared with the vehicle group.

Chamber assay (S1P and VEGF dependent). This assay has been described previously (13). Briefly, sterile porous Teflon chambers were filled with 0.8% agar (BBL Nr. 11849, Becton Dickinson, Meylan, France) containing heparin (20 units/mL) with or without growth factors: $VEGF_{165}$ (2 μg/mL; Tumor Center, Freiburg, Germany), or 5 μmol/L S1P (ANAWA Biomedical, Zürich, Switzerland). The chamber was implanted s.c. on the dorsal flank of female mice (MAG and NIH/Tif). Animals were treated with FTY720 (0.3 or 3 mg/kg orally), NVP-AAL151 or NVP-AAL149 (2.5 mg/kg i.v.) or NVP-AAL993 (100 mg/kg orally) 4 to 6 hours before chamber implantation and then once daily for a further 3 days. On the fourth day after implantation, animals were sacrificed, and the vascularized fibrous tissue formed around each implant carefully removed and weighed. Tissue samples were then homogenized in 1 mL of radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris-HCl (pH 7.2), 120 mmol/L NaCl, 1 mmol/L EDTA, 6 mmol/L EGTA, 1% (v/v) NP40, 20 mmol/L NaF, to which fresh 1 mmol/L phenylmethylsulfonyl fluoride and 1 mmol/L Na-vanadate were added], centrifuged, and filtered. The amount of hemoglobin present in the supernatant was determined by spectrophotometric analysis at 540 nm using the Drabkin reagent kit (Sigma hemoglobin #525, Sigma Chemical Co., Ltd., Poole, Dorset, England).

Tie-2 measurements. Tie-2 levels were determined using an ELISA method. Nunc (Naperville, IL) Maxsisorb 96-well plates were coated over night at 4°C with the capture antibody, anti-Tie-2 AB33 (UBI, Hauppauge, NY), with a concentration of 2 μg/mL (100 μL/well). Wells were washed in TPBS (Tween 80 PBS) and blocked by incubating with 3% Top-Block (Juro, Lucerne, Switzerland) for 2 hours at room temperature. After washing, 300 μg of protein lysates were incubated for 2 hours before further washing and addition of a complex of detection antibody, goat anti-mouse Tie-2 (R&D Systems, Minneapolis, MN; 0.5 μg/mL) and alkaline phosphate conjugated to monoclonal antibody (mAb) anti-goat (Sigma, St. Louis, MO; diluted 1:6,000) in TPBS + 0.1%Top-Block for 1 hour at room temperature. After washing, Tie-2 antibody complexes were detected by incubating with *p*-nitrophenyl phosphate (Sigma, tablets) and reading absorbance with an ELISA reader at 405 nm.

Recombinant human extracellular domain of Tie-2 fused to the constant regions of human IgG1 (sTie-2Fc) dissolved in RIPA buffer was used as standard in a concentration range from 0.1 to 300 ng/well (Tie-2Fc was a kind gift from Georg Martiny-Baron, Novartis).

VEGF-induced microvascular permeability. Heparin immobilized acrylic beads (50-100 μm in diameter) were incubated overnight with PBS/O or a solution of 1 μg VEGF in 10 μL PBS/O. Subsequently, the beads were implanted s.c. in both ears of female MAG mice (8-10 beads per ear). Vascular permeability of the newly formed vessels was visualized after 2 days using Evans blue dye (2%, 10 ml/kg) that was injected i.v. 5 minutes before sacrifice. Measurements of the dye extravasation area (mm^2) were carried out using pixel-based threshold in a computer-assisted image analysis software (KS-400 3.0 imaging system, Zeiss, Jena, Germany). Mice were treated with FTY720 (0.3 and 3 mg/kg orally) or PTK/ZK (100 mg/kg orally) 2 hours before Evans blue injection.

Tumor model. The syngeneic B16/BL6 murine melanoma model, previously shown to be responsive to antiangiogenic therapy (13), was used to evaluate the antitumor activity of FTY720. B16BL6 melanoma cells (kind gift from Dr. Isaiah J. Fidler, Texas Medical Center, Houston, TX) were cultured until confluency. Tumor cells (1 μL, 5×10^4/μL) was injected intradermally into the dorsal pinna of both ears of syngeneic female C57BL/6 mice. Measurements of primary tumor area (mm^2) were carried out on days 7, 14, and 21 after tumor cell inoculation using computer-assisted image analysis software (KS-400 3.0 imaging system, Zeiss) and a specially designed macro. From days 7 to 21, mice were treated orally once daily with either vehicle PEG300 (5 mL/kg), FTY720 (3 mg/kg), PTK/ZK (100 mg/kg), or a combination of the two compounds at the above doses. Mice were sacrificed on day 21, and cranial lymph node metastases were weighed and then frozen in ornithine carbamyl transferase cryofreezing medium for histologic analysis.

Histologic analysis and lectin perfusion. Lectin (200 μL) from *Ricinus communis* agglutinin-1, FITC conjugated (Vector Labs, Burlingame, CA), at a concentration of 1 μg/μL in sterile 0.9% NaCl was injected into the tail vein of a B16/BL6 melanoma bearing C57/B6J mouse. The mouse was left for 30 minutes before sacrifice and tumor excision. Frozen sections (12 μm) were subjected to immunohistochemical analysis as described previously (14). Antibodies used were rat anti-mouse CD31 (BD PharMingen, San Diego, CA; diluted 1:600 in PBS), rabbit anti-mouse active caspase 3 (Oncogene, Uniondale, NY; diluted 1:10 in PBS), and rabbit anti mouse Ki67 (Neomarkers, Fremont, CA; diluted 1:200 in PBS). Secondary antibodies were goat anti-rabbit or goat anti-rat ALEXA 568 or 488 (Molecular Probes, 1:400).

Fluorescence-activated cell sorting analysis. Mice bearing B16/BL6 melanoma tumors were treated daily for 7 days (days 7-14) with FTY720 (3 mg/kg orally), PTK/ZK (100 mg/kg orally) or the combination of both drugs. At the end of treatment, mice were sacrificed, cranial lymph node metastases were surgically removed and minced into small pieces, and a single-cell suspension was formed by collagenase/dispase treatment and subjected to fluorescence-activated cell sorting (FACS) analysis. Due to their small size, tumor cells were detected as a distinct population in the scatter plot and can be accordingly gated and enumerated.

HUVEC cells were treated with vehicle alone or FTY-P (10 and 100 nmol/L) for 30 minutes and were analyzed by FACS for VEGFR2 levels on the cell surface. Cells were trypsinized, washed twice with PBS containing 10% (v/v) fetal bovine serum (FBS), and incubated 10 minutes on ice before the addition of mouse mAb anti-VEGFR2/KDR 1495.12.14 (13) antibody developed within our laboratories in Novartis (2 μg mAb/10^6 cells). After 1 hour of incubation on ice, cells were washed twice in PBS plus 10% FBS, and RPE-labeled anti-mouse was added to the cells (BD PharMingen). FACS acquisition and analysis were done on a FACSCalibur using Cell Quest Software (Becton Dickinson).

Analysis of $S1P_1$ receptor localization. HEK293 cells expressing $S1P_1$-GFP fusion protein (15) were used in this assay. Cells were serum starved for 2 hours and treated with indicated concentrations of S1P or FTYP or

structural analogues as described (10). At specific times, cells were fixed and imaged on a Zeiss 510 confocal microscope as previously described (15).

Blood cell counts. Blood analysis was done using a commercially available blood analyzer ABC VET 16 (Axon Lab AG, Baden-Dättwil, Switzerland). Blood was collected in EDTA tubes via the vena cava inferior immediately after sacrifice of the mice by CO_2 inhalation. An aliquot (12 μL) of EDTA-blood obtained from all groups of mice treated for 21 days was taken 24 hours after the last dose and analyzed for WBC, RBC, platelets, lymphocytes, monocytes, and granulocytes.

Proliferation assay. Subconfluent B16/BL6 melanoma cells were seeded at a density of 3×10^3 per well into 96-well plates and incubated at 37°C and 5% CO_2 in growth medium (10% FBS in MEM EBS, Amimed). After another 24 hours, the medium was renewed and PTK787/ZK222584, FTY720, FTY-P, or vehicle were added. After 8 hours of incubation, bromodeoxyuridine (BrdUrd) labeling solution was added, and cells were incubated a further 16 hours before fixation, blocking, and addition of peroxidase-labeled anti-BrdUrd antibody. Bound antibody was then detected using 3,3′ 5,5′-tetramethylbenzidine substrate, which forms a colored reaction product that is quantified spectrophotometrically at 450 nm.

Apoptosis assay. Subconfluent B16/BL6 melanoma cells were seeded at a density of 3×10^3 per well into 96-well plates and incubated at 37°C and 5% CO_2 in growth medium (10% FCS in MEM EBS, Amimed). After another 24 hours, the medium was renewed and PTK787/ZK222584, FTY720, FTY-P, or vehicle was added. Twenty-four hours later, induced cell death was measured photometrically through determination of cytoplasmic histone-associated DNA fragments, upon instructions of the supplier from the kit (Cell Death Detection ELISA^{PLUS}, Roche, Indianapolis, IN).

Western analysis. HUVECs at 80% confluency in EBM medium containing 0.5% (v/v) FBS were incubated with 500 nmol/L of either FTY720, FTY-P, or PTK/ZK for either 20 minutes or 1 hour. For the last 10 minutes of the incubation time, some of the HUVECs were stimulated with VEGF 10 ng/mL, and cells were subsequently lysed with RIPA buffer [50 mmol/L Tris-HCl (pH 7.2), 120 mmol/L NaCl, 1 mmol/L EDTA (pH 8), 6 mmol/L EGTA (pH 8.5), 1% (v/v) NP40, and 20 mmol/L NaF]. Ten micrograms of lysates were run on a 10% SDS gel and blotted. The gel was first probed with p44/42 mitogen-activated protein kinase (MAPK) antibody (Cell Signaling, Beverly, MA) and then stripped and reprobed with an α-tubulin antibody (Neomarkers) to control for equal loading.

Statistical analyses. Results are presented as mean ± one SE. Between-group differences used one-way ANOVA or two-way ANOVA employing Holm-Sidak tests for post hoc comparisons (either pairwise or versus controls). In some cases, the data were normalized by taking log10 before statistical analyses. For all tests, the level of significance was set at $P < 0.05$. Statistical calculations were done using SigmaStat 3.1 (Jandel Scientific, San Rafael, CA).

Results

FTY720 inhibits S1P-driven angiogenesis. Because S1P is a proangiogenic factor, we tested the effects of the S1PR modulator FTY720 (and analogues) in an S1P-driven angiogenesis agar chamber model (see Fig. 1*A* and *C*). We carried out the experiment in the same manner as the VEGF-driven chamber model previously used to characterize the VEGFR inhibitor PTK787/ZK222584 (PTK/ZK; ref. 13) but substituting S1P for VEGF. The effects of FTY720 at 3 and 0.3 mg/kg, an analogue NVP-AAL151 and its inactive enantiomer NVP-AAL149 both at 2.5 mg/kg, and a VEGF receptor tyrosine kinase inhibitor NVP-AAL993 at 100 mg/kg were assessed. The mice received a single administration of vehicle or compound 4 to 6 hours before implantation and were subsequently treated once daily for 3 days before explanting the chamber. In this time, a new blood vessel–rich tissue is formed around the implanted chamber. This tissue was removed, weighed, and analyzed for total amount of hemoglobin (a measure for vascularity and hemorrhage) and Tie-2 protein (indicative of endothelial cell amount and therefore vascularity only) in the tissue.

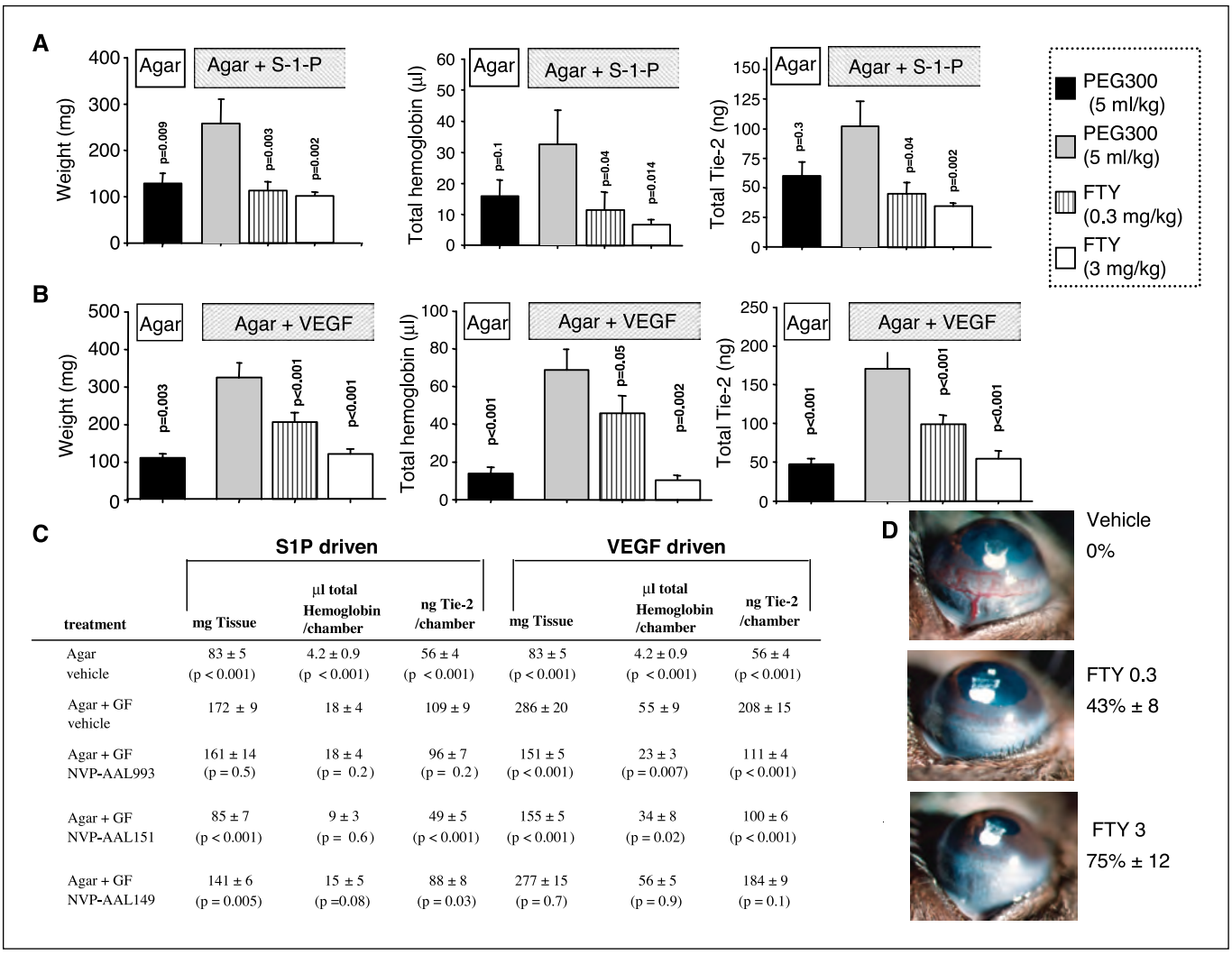
S1P was overall a weaker promoter of the indices of angiogenesis in this chamber model compared with VEGF (Fig. 1). FTY720 at doses of 0.3 and 3 mg/kg reduced the weight of newly formed tissue and its hemoglobin and blood vessel content in the S1P-driven agar implant model (Fig. 1*A*). NVP-AAL151 also functioned as an inhibitor in this model, whereas NVP-AAL149 was considerably weaker. As expected, the VEGFR tyrosine kinase inhibitor NVP-AAL993 did not exert any influence on the S1P-driven angiogenesis model (Fig. 1*C*). These results show that FTY720 and NVP-AAL151 are able to inhibit S1P-driven angiogenesis *in vivo*.

FTY720 inhibits VEGF-driven angiogenesis. We further tested if FTY720 and its analogues at the same doses as above were able to inhibit VEGF-driven angiogenesis using the s.c. implant model (Fig. 1*B* and *C*). VEGF-filled agar chambers (Fig. 1*B, gray columns*) produced increased weight as well as amount of hemoglobin and Tie-2 in the newly formed tissue compared with an agar implant with no growth factor (Fig. 1*B, black columns*). FTY720 at 3 and 0.3 mg/kg inhibited the VEGF-dependent increased weight ($P < 0.001$ versus VEGF control) and Tie-2 ($P < 0.001$ versus VEGF control) and hemoglobin ($P < 0.05$ versus VEGF control) content of the tissue. There was a tendency for the 3 mg/kg dose to be more active than the 0.3 mg/kg dose in all of the variables evaluated. However, this only reached statistical significance with hemoglobin content. The immunosuppressive properties of FTY720 were evident by the reduced number of WBC, specifically lymphocytes, circulating in the blood (data not shown). Platelets, granulocytes, and RBC were unaffected (data not shown).

With the exception of NVP-AAL149 (the inactive enantiomer that is not phosphorylated by sphingosine kinase; see refs. 9, 10), all compounds were able to inhibit the increase in weight of the newly formed tissue, hemoglobin, and Tie-2 content in this model (Fig. 1*C*). NVP-AAL149 was significantly less active than either NVP-AAL151 or NVP-AAL993 in terms of impairing tissue accumulation and Tie-2 levels ($P < 0.001$), hemoglobin content of the tissue (versus NVP-AAL151, $P = 0.013$; versus NVP-AAL993, $P = 0.005$). Interestingly, in this VEGF-driven angiogenesis model, the VEGFR inhibitor NVP-AAL993 and the S1PR signaling modulator NVP-AAL151 were not statistically different in their inhibitory effects (mg tissue, $P = 0.9$; hemoglobin levels, $P = 0.3$; Tie-2 levels, $P = 0.5$). These results show that at doses producing leukopenia, FTY720 and its analogues have potent antiangiogenic activity in a well-established *in vivo* angiogenesis model driven by two distinct proangiogenic factors.

We also tested the ability of FTY720 to block neoangiogenesis in the mouse corneal pocket assay, a widely used angiogenesis model. In this model (12), VEGF pellets were surgically implanted into avascular corneas of C57BL/6 mice to induce a robust angiogenesis response (Fig. 1*D*). Twenty-four hours after implantation, mice were treated with either vehicle alone or FTY720 at 0.3 or 3 mg/kg orally once per day. The eyes were examined on postoperative days 3 through 6, and the induced vascular response was measured. Oral treatment with FTY720 caused a marked (versus controls: 0.3 mg/kg FTY720, $P = 0.002$; 3 mg/kg FTY720, $P < 0.001$) and apparently dose-dependent inhibition of new blood vessel formation (3 mg/kg FTY720 superior to 0.3 mg/kg, $P = 0.02$).

FTY720 reduces leakiness of blood vessels. Because blockade of the hemoglobin response in the chamber model may reflect an effect on vascular permeability rather than an effect on new vessel



treatment	S1P driven: mg Tissue	S1P driven: µl total Hemoglobin /chamber	S1P driven: ng Tie-2 /chamber	VEGF driven: mg Tissue	VEGF driven: µl total Hemoglobin /chamber	VEGF driven: ng Tie-2 /chamber
Agar vehicle	83 ± 5 (p < 0.001)	4.2 ± 0.9 (p < 0.001)	56 ± 4 (p < 0.001)	83 ± 5 (p < 0.001)	4.2 ± 0.9 (p < 0.001)	56 ± 4 (p < 0.001)
Agar + GF vehicle	172 ± 9	18 ± 4	109 ± 9	286 ± 20	55 ± 9	208 ± 15
Agar + GF NVP-AAL993	161 ± 14 (p = 0.5)	18 ± 4 (p = 0.2)	96 ± 7 (p = 0.2)	151 ± 5 (p < 0.001)	23 ± 3 (p = 0.007)	111 ± 4 (p < 0.001)
Agar + GF NVP-AAL151	85 ± 7 (p < 0.001)	9 ± 3 (p = 0.6)	49 ± 5 (p < 0.001)	155 ± 5 (p < 0.001)	34 ± 8 (p = 0.02)	100 ± 6 (p < 0.001)
Agar + GF NVP-AAL149	141 ± 6 (p = 0.005)	15 ± 5 (p =0.08)	88 ± 8 (p = 0.03)	277 ± 15 (p = 0.7)	56 ± 5 (p = 0.9)	184 ± 9 (p = 0.1)

Figure 1. Antiangiogenic effect of FTY720. *A,* effect of FTY720 (0.3 or 3 mg/kg orally) or vehicle (PEG300, 100% 5 mL/kg orally) on an S1P-driven chamber model (5 µmol/L). Weight (*left*), total hemoglobin (*middle*), and Tie-2 levels (*right*) of the tissue are plotted. From two independent experiments were pooled (10-12 mice). *Columns,* mean; *bars,* SE. The statistical significance of inhibition compared with vehicle-treated S1P containing chambers was determined using one-way ANOVA with post hoc Holm-Sidak tests. *B,* effect of FTY720 (0.3 or 3 mg/kg orally) or vehicle (PEG300, 100% 5 mL/kg orally) on the VEGF-driven chamber model (2 µg/mL). Weight (*left*), total hemoglobin (*middle*), and Tie-2 levels (*right*) of the tissue are plotted. From two independent experiments were pooled (10-12 mice). *Columns,* mean; *bars,* SE. Statistical significance of inhibition was determined using one-way ANOVA with post hoc Holm-Sidak tests. *C,* treatment of mice with NVP-AAL993 (100 mg/kg orally), NVP-AAL149 (2.5 mg/kg i.v.), NVP-AAL151 (2.5 mg/kg i.v.), or vehicle (PEG300, 100% 5 mL/kg orally) in an S1P-driven (5 µmol/L) or VEGF-driven (2 µg/mL) agar chamber model (see *top*). The animals were sacrificed for measurement of the vascularized chamber-adherent tissues (weight and hemoglobin and Tie-2 content) 24 hours after the last dose (n = 10-12 per group, pooled data from two independent experiments per dose). *GF,* growth factor. Statistical significance of inhibition was determined using one-way ANOVA with post hoc Holm-Sidak tests. *D,* C57/BL6J mouse corneal pocket assay. Photomicrographs showing vascularization induced by VEGF-implanted pellets after 6 days of treatment with vehicle (5% glucose in water), 0.3 mg/kg FTY720, or 3 mg/kg FTY720. *Numbers,* % inhibition of blood vessel growth (mean ± SE). Both the high-dose FTY720 group (P < 0.001) and the low-dose group (P = 0.002) were statistically significantly different from controls (one-way ANOVA with post hoc Holm-Sidak test). The experiment was done two separate times. Representative experiment. Quantitative data pooled from both experiments (n = 6 per group).

formation, we investigated the effect of FTY720 in a specific assay for vascular permeability (Fig. 2). For this purpose, VEGF-soaked beads were implanted s.c. into one ear of a mouse, which produces new but leaky and torturous vessel formation over a period of 2 days. The other ear of the same mouse is implanted with PBS soaked beads as a control. After 2 days, Evans blue is injected i.v., and images of the ears are taken. Blood vessel leakiness is assessed initially by visual inspection (Fig. 2*A*) and by image analysis determination of the area of extravasated Evans blue dye (Fig. 2*A* and *B*). Vehicle, PTK/ZK (100 mg/kg), or 0.3 or 3 mg/kg FTY720 were administered 2 hours before Evans blue injection. Two-way ANOVA indicated that VEGF increased vessel leakiness compared with that produced by control beads (P < 0.001), and both compounds alone inhibited both basal and VEGF-induced vascular permeability changes. At both 0.3 and 3 mg/kg, FTY720 inhibited basal leakiness (likely to be caused by wounding during implantation or inflammatory processes associated with the intracorporal presence of a foreign body; Fig. 2*A* and *B*) as well as VEGF-induced leakiness of the vessels. As expected, the two VEGF receptor tyrosine kinase inhibitors NVP-AAL993 (not shown) and PTK/ZK also effectively reduced leakiness of the vasculature in this model (Fig. 2). When comparing (two-way ANOVA) the effect of treatment in the absence of VEGF stimulus, PTK/ZK or FTY720 at 0.3 or 3 mg/kg inhibited vascular leakiness, but these treatments

were not significantly different from each other (all *P*s < 0.05). The presence of VEGF clearly promoted vascular permeability. Within the VEGF-treated groups, PTK/ZK and 0.3 mg/kg FTY720 impaired vascular permeability by (P = 0.022 and P < 0.001 versus controls), and 3 mg/kg FTY720 produced an effect greater than either of the other two active treatments (Fig. 2*B*). These results indicate that part of the observed pharmacologic antiangiogenic activity of FTY720 may be due to abrogation of the increases in vascular permeability caused by VEGF.

FTY720 pretreatment inhibits S1P-stimulated migration and calcium mobilization *in vitro*. Because FTY720 was recently shown to act as a functional antagonist on $S1P_1$ receptors on T cells, inhibiting their egress from lymph nodes (16), we assessed whether FTY720 can also modulate the response of endothelial cells to S1P. We first examined the effect of FTY720, FTY-P, NVP-AAL151, and NVP-AAL149 on S1P-driven endothelial migration *in vitro* (data not shown). Compounds were preincubated with HUVEC for 30 minutes before the cells were allowed to migrate towards S1P (500 nmol/L) in the continuing presence of compounds. FTY720, FTY-P, and NVP-AAL151 were all able to significantly inhibit S1P-induced HUVEC migration with IC_{50} values of 6.5, 2.5, and 7.4 nmol/L, respectively, whereas NVP-AAL149 was inactive at all doses tested (>250 nmol/L). Because the prodrug FTY720 was active in this assay, this suggested that the 4-hour incubation time of the assay was sufficient for conversion of FTY720 to FTY-P by sphingosine kinase and for the latter form to subsequently inhibit the S1P-mediated migration of cells (17).

Activation of S1PR by S1P causes G-protein–coupled activation and mobilization of calcium from the endoplasmic reticulum (9, 18). We tested FTY720 and its analogues in this assay to see if they could antagonize S1P-induced Ca^{2+} mobilization (data not shown). The compounds were preincubated with HUVEC for 3 hours before the assay to allow the prodrugs to be phosphorylated by sphingosine kinase. FTY-P, FTY720, and NVP-AAL151 were all able to block the S1P-driven calcium mobilization with IC_{50} values of 55, 164, and 156 nmol/L, respectively, whereas NVP-AAL149 was unable to inhibit the S1P response (>34.4 μmol/L). The IC_{50} differences observed between the migration assay and Ca^{2+} mobilization are probably due to the longer incubation period (FTY720 converting to FTY-P) in the migration assay as well as the difficulty of inhibiting the amplification of signaling in the Ca^{2+} mobilization assay.

FTY-P internalizes $S1P_1$. To investigate the mechanism by which FTY720 and its analogues are modulating the S1P-driven responses, we incubated HEK293 cells stably expressing an $S1P_1$-GFP fusion protein (15) with 10 nmol/L of either S1P or FTY-P for 60 minutes and analyzed the localization of $S1P_1$ by confocal fluorescence imaging. $S1P_1$-GFP is normally expressed on the cell surface (Fig. 3*A*). Addition of S1P at 10 nmol/L did not affect the localization of this receptor; however, addition of FTY-P at the same concentration resulted in the internalization of $S1P_1$ as detected by the punctated endosomal appearance for $S1P_1$. An additional experiment was designed to investigate recycling of the receptor back to the surface of HEK293 cells (Fig. 3*B*). In this case, 100 nmol/L of S1P or FTY-P were added to the cells. This high dose of ligand resulted in receptor internalization in both cases after 60 minutes, although a significantly greater internalization was observed with the FTY-P treatment. The ligands were subsequently washed out, and the cells were allowed to recover for 60 minutes.

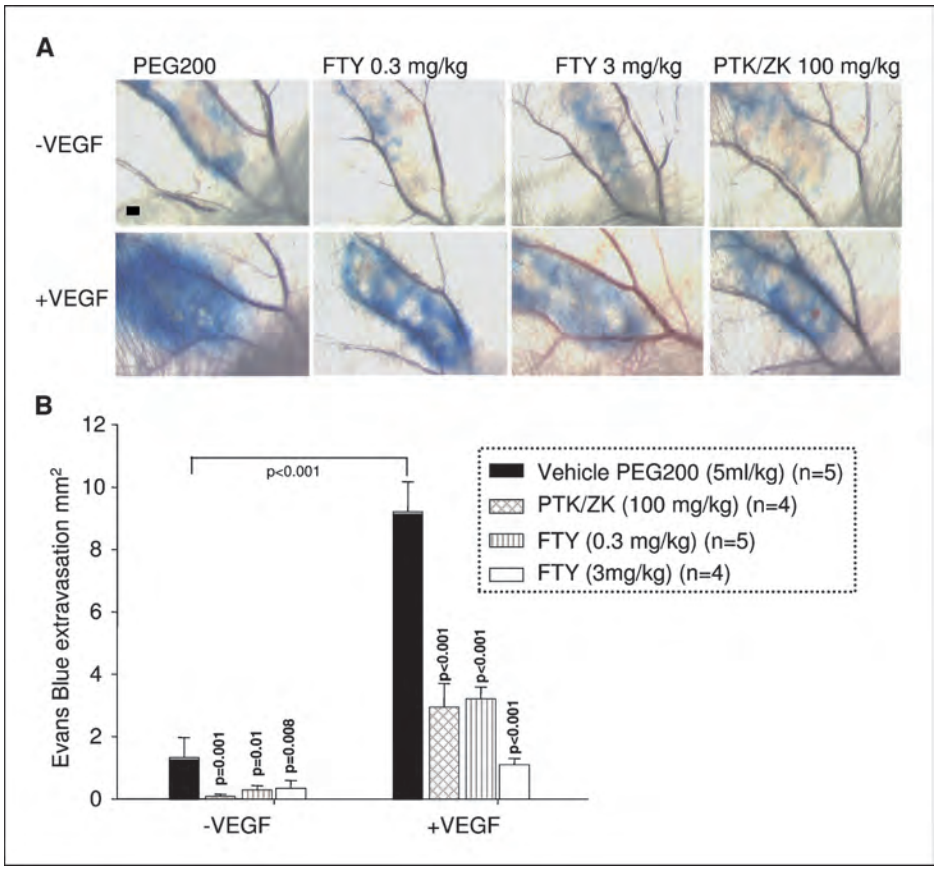


Figure 2. FTY720 reduces leakiness of vessels. *A,* photomicrographs showing leakage of Evans blue from vessels with implanted PBS-soaked beads (*top*) and VEGF-soaked beads (*bottom*). Treatment with FTY720, PTK/ZK, or vehicle. Bar, 1 mm. This experiment was done twice. Representative experiment. *B,* measurements of the dye extravasation area (mm^2) were carried out using computer-assisted image analysis software. *Columns,* mean; *bars,* SE. *P*s are from one-way ANOVA with post hoc Holm-Sidak tests.

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