sFLT01: A Novel Fusion Protein with Antiangiogenic Activity

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Abstract

sFLT01 is a novel fusion protein that consists of the VEGF/PlGF (placental growth factor) binding domain of human VEGFR1/Flt-1 (hVEGFR1) fused to the Fc portion of human IgG1 through a polyglycine linker. It binds to both human VEGF (hVEGF) and human PlGF (hPlGF) and to mouse VEGF (mVEGF) and mouse PlGF (mPlGF). In vitro, sFLT01 inhibited the proliferation of human umbilical vein endothelial cells and pericytes stimulated by either hVEGF or hPlGF. In vivo, sFLT01 had robust and significant antitumor activity in numerous preclinical subcutaneous tumor models including H460 non–small cell lung carcinoma, HT29 colon carcinoma, Karpas 299 lymphoma, MOLM-13 AML (acute myeloid leukemia), 786-O, and RENCA renal cell carcinoma (RCC). sFLT01 also increased median survival in the orthotopic RENCA RCC model. sFLT01 had strong antiangiogenic activity and altered intratumoral microvessel density, blood vessel lumen size and perimeter, and vascular and vessel areas in RCC models. sFLT01 treatment resulted in fewer endothelial cells and pericytes within the tumor microenvironment. sFLT01 in combination with cyclophosphamide resulted in greater inhibition of tumor growth than either agent used alone as a monotherapy in the A673 Ewing’s sarcoma model. Gene expression profiling indicated that the molecular changes in the A673 sarcoma tumors are similar to changes observed under hypoxic conditions. sFLT01 is an innovative fusion protein that possessed robust antitumor and antiangiogenic activities in preclinical cancer models. It is a dual targeting agent that neutralizes both VEGF and PlGF and, therefore, has potential as a next generation antiangiogenic therapeutic for oncology. Mol Cancer Ther; 10(3); 404–15. ©2011 AACR.

Introduction

The recognition that tumors require an active network of blood vessels for growth and metastasis generated the field of angiogenesis research. VEGF was identified as a major contributor to the angiogenesis process under both physiologic and pathologic conditions that include cancer (1). Multiple isoforms of VEGF and multiple splice variants of VEGF-A have been implicated in the progression of malignant disease. The various species of VEGF bind to any of several VEGF receptors and the neuropilin coreceptors on the cell surface. The development of antiangiogenic therapies targeting VEGF pathways followed their elucidation. Bevacizumab, a humanized monoclonal antibody that neutralizes human VEGF-A, was the first approved antiangiogenic drug for use with chemotherapy in metastatic colorectal cancer patients (2). Subsequently, sunitinib and sorafenib, small molecule multitargeted tyrosine kinase inhibitors, were approved for advanced renal cell carcinoma (RCC), a highly vascularized disease characterized by VEGF overexpression (3). However, although VEGF is a well-validated target, the overall survival benefit with the first-generation antiangiogenic therapies that inhibit VEGF pathways has been modest and there remains a need to improve upon the standard of care.

Placental growth factor (PIGF) is a member of the VEGF family and can form heterodimers with VEGF (4, 5). Several splice variants of PIGF exist that bind to VEGFR1/Flt-1 and/or neuropilin-1 (6, 7). PIGF angiogenic activity was shown in transgenic mice in which the overexpression of PIGF resulted in a substantial increase in vasculature including the number of vessels, branching points, size of the vessels, and increased vascular permeability (8). PIGF is overexpressed in multiple human malignancies including renal, breast, gastric, and lung cancers and brain tumors (9–15). It has potential as a prognostic biomarker in breast, colon, and lung cancers because higher PIGF circulating levels are associated with more aggressive disease (10, 15–17). The therapeutic potential of PIGF neutralization was shown with an anti-PIGF antibody that inhibited the growth of tumors in preclinical models of melanoma, pancreatic cancer, and colon carcinoma (18).

Serum PIGF levels increase following cancer therapy and it is one of several factors implicated in therapeutic
resistance, posttherapy angiogenesis, and tumor regrowth (19). PIGF was associated with early recurrence of hepatocellular carcinoma following radical resection and was upregulated in preclinical lung and colon adenocarcinoma tumors following radioimmunotherapy (20, 21). In colorectal cancer patients with metastatic disease, serum PIGF levels increased following the administration of bevacizumab in combination with chemotherapy and/or radiation (22, 23). VEGF-A and PIGF levels increased following sunitinib treatment in patients with bevacizumab-refractory metastatic RCC and in men with advanced prostate cancer who were treated with sunitinib (24, 25). Thus, PIGF may have a role in resistance to certain antiangiogenic therapies and its neutralization may overcome resistance to VEGF receptor inhibitors (26).

We have investigated the potential value of targeting both VEGF and PIGF for oncology, using sFLT01, a novel fusion protein that consists of the VEGF/PIGF binding domain of human VEGFR1/Flt-1 (hVEGFR1) fused to the Fc portion of human IgG1 through a polyglycine linker (9Gly; ref. 27). sFLT01 has a molecular weight of approximately 80 kDa and does not contain the extracellular domain 3 of VEGFR2 as aflibercept does. Intravitreal delivery of an AAV2 vector, encoding sFLT01 (AAV2.sFLT01), was efficacious in a gene therapy treatment of murine and nonhuman primate models of neovascularization (27, 28). sFLT01 functions as a soluble decoy receptor that binds human VEGF-A (hVEGF) and human PIGF (hPIGF). It also binds to mouse VEGF-A (mVEGF) and PIGF (mPIGF) and therefore in preclinical tumor models can target VEGF and PIGF secreted by the human malignant cells in xenografts, by syngeneic neoplastic cells, and/or by the host stroma. We have explored the role of both angiogenic growth factors on the cellular components that comprise blood vessels, endothelial cells (EC), and pericytes. Our results show that hVEGF and hPIGF promote not only EC proliferation but also pericyte stimulation in vitro. This effect was diminished by exposure of the cells to sFLT01. In vivo, sFLT01 treatment resulted in fewer ECs and pericytes in subcutaneous RECA tumors. The strategy of dual targeting of EC and pericytes has shown benefit in preclinical models (29).

sFLT01 as a single agent had significant antitumor activity against a wide range of SC xenograft tumors including the H460 non–small cell lung carcinoma (NSCLC), HT29 colon carcinoma, Karpas 299 lymphoma, MOLM-13 AML (acute myeloid leukemia), and 786-O RCC. It was also efficacious in the syngeneic RECA RCC model, with activity against both SC and orthotopic tumors. sFLT01 in combination with cyclophosphamide resulted in a greater inhibition of tumor growth in the A673 Ewing’s sarcoma model than either drug used alone as a monotherapy. The antiangiogenic activity of sFLT01 was evident in the 786-O and RECA models, with significant alterations in microvessel density (MVD), lumen size and vessel perimeter, and other vascular parameters. Double immunofluorescence methods detected sFLT01 intratumorally both in the vicinity of the vasculature and in necrotic areas in A673 tumors. Further evidence of the antiangiogenic properties of sFLT01 was apparent following molecular profiling of A673 tumors from mice treated with sFLT01, which revealed that the changes in gene expression were similar to those observed under hypoxic conditions.

The results presented here suggest that sFLT01 may have potential as an antiangiogenic therapeutic. sFLT01 was active in a wide variety of tumor models that represent both solid tumors types and hematologic malignancies. It has dual targeting properties that may translate into greater efficacy in patients in whom both VEGF and PIGF are upregulated or in cases in which PIGF contributes to drug resistance. sFLT01 is a unique and innovative molecule with a structure and mechanism of action which differentiates it from other antiangiogenic drugs that are currently approved for oncology indications.

Materials and Methods

sFLT01 protein

CHO DXB11 cells were transfected with an expression vector containing the coding sequence of sFLT01 (27), using the Lipofectamine 2000 reagent (Invitrogen). Transfected cells were selected with increasing concentrations of methotrexate in MEM alpha-no ribonucleosides or deoxyribonucleosides (Invitrogen) containing 10% dialyzed FBS (Invitrogen). The transfected pool was grown in 850-cm² roller bottles in 200 mL of selection medium. When the cells reached confluence, the selection medium was removed and replaced with IS CHO CD medium (Irvine Scientific). The conditioned medium was harvested 3 to 4 days later, filtered through a 0.2-μm pore filter, and loaded onto a Protein A column that was preequilibrated with 0.7 mol/L NaCl, 20 mmol/L Tris, pH 7.0. The column was washed with equilibration buffer and eluted with 50 mmol/L glycine, pH 2.5. The eluted peak fraction containing sFLT01 protein was adjusted to pH 6.5–6.7 with 0.3 mol/L sodium phosphate, dibasic. The sFLT01 was concentrated to 10 mg/mL using a size 4, 30-kDa cutoff cartridge (GE Healthcare Life Sciences) and diafiltration with PBS (Invitrogen).

Binding assays

Nunc Maxisorp 96-well plates were coated overnight at 4°C with 10 μg/mL of recombinant hVEGF-A (rVEGF-A) or recombinant mVEGF-A (rmVEGF-A; R&D Systems) in PBS. The plate was washed once and blocked with 1% bovine serum albumin (BSA) in PBST (PBS, 0.05% Tween-20) for 1 hour at room temperature and washed 3 times with PBST. sFLT01 was serially diluted in PBS and added to the wells, and the plate was incubated for 2 hours at room temperature, followed by a wash with PBST. A peroxidase-conjugated goat anti-human Fc antibody (1/10,000; R&D Systems) was added for 1 hour at room temperature. Plates were washed 6 times with PBST followed by the addition of TMB substrate and a stop
solution (1N sulfuric acid). The plate was read at 450 nm, and EC50 values were calculated using nonlinear regression analysis.

Nunc Maxisorp 96-well plates were coated with sFLT01 (100 μg/mL, 10 μg/well) in PBS overnight at 4°C. The plates were washed once and blocked with 1% BSA in PBST for 1 hour at room temperature. Recombinant hPIGF (rhPIGF) and recombinant mPIGF (rmPIGF; R&D Systems), were serially diluted in PBS and added to the wells, and the plates were incubated for 2 hours at room temperature. The plates were washed 3 times with PBST incubated with peroxidase-conjugated anti-PIGF antibodies (R&D Systems) for 1 hour. The plate was washed 6 times with PBST followed by the addition of TMB substrate and a stop solution (1N sulfuric acid). The plate was read at 450 nm, and EC50 values were calculated using nonlinear regression analysis.

Proliferation assays

Human umbilical vein endothelial cells (HUVEC; Lonza) were plated in triplicate overnight (2 x 10^3 cells/well) in DMEM media/5% FBS (Invitrogen) in 96-well plates. rhVEGF-A (20 ng/mL) or rhPIGF (50 ng/mL; R&D Systems, and sFLT01 (40–200 ng/mL were preincubated for 5 minutes at room temperature before addition to the cells. Cells were allowed to grow for 3 days and then assayed by Titer Glo Luminescent Cell Viability Assay (Promega).

Human pericytes (Sciencell Research Labs) were plated overnight in triplicate (4 x 10^3 cells/well) in pericyte basal media/2% FBS (Sciencell Research Labs) in 96-well plates. rhVEGF-A (10 ng/mL) or rhPIGF (10 ng/mL; R&D Systems, and sFLT01 (40–200 ng/mL were preincubated for 5 min before the addition to the cells. Cells were allowed to grow for 4 days and then assayed by Titer Glo Luminescent Cell Viability Assay (Promega). Cell lines were not authenticated by the authors.

In vivo tumor models

The efficacy of sFLT01 was evaluated in human xenograft and mouse syngeneic tumor models. All procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee in accordance with the Federal Animal Welfare Act (9 CFR, 1992) and were conducted in a AAALAC-accredited facility. For the human H460 NSCLC, HT29 colon carcinoma, MOLM-13 AML, and 786-O RCC models, nude (nu/nu) mice (Harlan) were implanted subcutaneously with 9 x 10^6 cells mixed 1:1 with Matrigel and were implanted in the subrenal capsule of the kidney with 2 x 10^6 cells in PBS. Cell lines were not authenticated by the authors.

Treatments were initiated when SC tumors reached 100 to 200 mm^3 or on day 5 postorthotopic implantation of RENCA cells. Animals were pair-matched into treatment or control groups (n = 8–14). sFLT01 was intraperitoneally injected twice per week for 4 to 7 weeks in the H460 NSCLC (25 mg/kg), HT29 colon carcinoma (10 mg/kg), Karpas 299 lymphoma (25 mg/kg), MOLM-13 AML (25 mg/kg), 786-O RCC (25 mg/kg), RENCA RCC SC (5 or 25 mg/kg), RENCA RCC orthotopic (25 mg/kg), and A673 sarcoma (5 mg/kg) models. For combination regimens, mice bearing A673 Ewing’s sarcoma tumors were treated with cyclophosphamide (125 mg/kg) once per week by intraperitoneal injection along with sFLT01 (5 mg/kg) administration as described earlier. Body weights were determined twice per week in all experiments. Tumor volumes were calculated using the formula (w^2 x l)/2 where w is the width of the tumor and l is the length of the tumor. Individual tumor measurements were taken with calipers twice weekly. Error bars in the tumor volume data represent SEM.

Immunohistochemistry

786-O tumors were collected on day 23, a day after the fourth dose of sFLT01 or vehicle and when tumors were approximately 225 mm^3 in the sFLT01 treatment group and 500 mm^3 in the control group. RENCA tumors were collected on day 11, a day after the second dose of sFLT01 or vehicle and when tumors were approximately 100 mm^3 in the sFLT01 treatment group and 130 mm^3 in the control group. All procedures were carried out at room temperature. 786-O and RENCA RCC SC tumors were fixed in 10% neutral buffered formalin and embedded in paraffin. Five-micrometer sections were incubated with protease-K (DAKO) for 10 minutes, blocked with 10% goat serum/TBS for 10 minutes, and incubated with 20 μg/mL rat anti-mouse CD31 antibody (Jackson Immunochemicals) was applied for 30 minutes, followed by 2 washes as described earlier. ABC-Elite peroxidase (Vector Laboratories) was applied for 20 minutes, and after 2 washes, the DAB (DAKO) Chromogen was applied for detection for 2 minutes. Tumors were counterstained with hematoxylin and whole image scans were generated at 20x magnification, using the Aperio SCANSCOPE XT scanning system. MVD represents the total number of CD31+ vessels/mm^2 of tissue area as quantified using the Aperio MVD algorithm. Additional morphometric measurements such as vessel lumen size and perimeter and vascular and vessel areas were also generated using the Aperio MVD algorithm and are reported in μm and μm^2, respectively.

RENCA tumors were collected on day 11, a day after the second dose of sFLT01 or vehicle, flash frozen in an optimum cutting temperature compound, cut into 4-
5-µm sections, and fixed in 10% zinc formalin (Electron Microscopy Sciences) for 10 minutes. After a 10-minute block in 10% goat serum/TBS, 10 µg/mL rat anti-mouse CD31 antibody (BD Biosciences) and 5 µg/mL rabbit anti-mouse NG2 antibody (Chemicon) were applied for 1 hour to detect ECs and pericytes, respectively. After 2 washes, secondary goat anti-rat-Cy3 and goat anti-rabbit-Cy2 antibodies (Jackson Immunohchemicals) at 5 µg/mL were applied simultaneously for detection for 20 minutes. Images were captured at 10× magnification with a Hamamatsu-100 camera and imported into Metamorph image analysis software (Molecular Devices). For each image, threshold was set to identify and measure the NG2-positive pericytes. The data are reported as the number of pericytes per mm².

A673 tumors were collected one day after the third (day 11) or sixth (day 21) dose of sFLT01 or saline, flash frozen in an optimum cutting temperature compound, and 4- to 5-µm tumor sections were fixed in 10% zinc formalin (Electron Microscopy Sciences) for 10 minutes. A rabbit anti-VEGFR2 antibody that cross-reacts with human and mouse VEGFR2 [1/1,000; Upstate (Millipore)] was applied for 60 minutes. After 2 washes, 5 µg/mL goat anti-human IgG CY2-labeled antibody (Jackson Immunohchemicals) and 2.5 µg/mL goat anti-rabbit-CY3 antibody (Jackson Immunohchemicals) were applied to the tissue sections for 30 minutes to allow simultaneous detection of VEGFR2 and sFLT01.

**Statistical analysis**

*In vitro* data are expressed as mean ± SD. Comparisons between the treatment and control groups were made using Student’s *t* test. Tumor volumes are expressed as mean ± SEM and were analyzed by ANOVA. Kaplan–Meier survival curves were analyzed by the log-rank test. Graphpad Prism5.0 (Graphpad Software, Inc.) was used for analysis. *P* < 0.05 was considered statistically significant.

**Results**

The ability of sFLT01 protein, expressed by transfected HEK293 cells into conditioned medium, to bind and neutralize hVEGF-A was shown previously *in vitro* in binding and HUVEC proliferation assays (27). The *in vitro* binding properties of recombinant sFLT01 protein were characterized in ELISA-based assays. sFLT01 at increasing concentrations was added to wells coated with rhVEGF-A or rmVEGF-A. The bound sFLT01 was quantified using a primary anti-Fc antibody and secondary peroxidase-conjugated antibody. The resulting EC₅₀ values for rhVEGF and rmVEGF were 0.132 and 0.0152 nmol/L, respectively (Fig. 1A). To measure the binding affinity of sFLT01 to rhPlGF and rmPlGF, increasing concentrations of each growth factor were added to wells coated with sFLT01. The bound rhPlGF or rmPlGF was measured by ELISA-based methods. The EC₅₀ values were calculated to be 0.300 nmol/L for hPlGF and 0.099 nmol/L for mPlGF (Fig. 1B). These data show the cross-species reactive property of sFLT01.

The *in vitro* activity of sFLT01 was evaluated in cell-based proliferation assays, using HUVEC or human pericytes. Both rhVEGF-A and rhPlGF stimulated HUVECs to proliferate, which was inhibited by sFLT01 in a concentration-dependent manner and generated EC₅₀ values of approximately 100 and 120 ng/mL, respectively (Fig. 1C). Similarly, rhVEGF-A and rhPlGF enhanced the proliferation of pericytes. sFLT01 inhibited pericyte proliferation stimulated by hVEGF-A or PlGF producing EC₅₀ values of approximately 110 and 160 ng/mL, respectively (Fig. 1D). Thus, sFLT01 can affect both cellular components that form growing vasculature, ECs, and pericytes, whether the cells are stimulated by rhVEGF-A or rhPlGF.

Robust antitumor efficacy of sFLT01 was shown in multiple xenograft models. Vehicle or sFLT01 (10 or 25 mg/kg) was delivered twice per week by intraperitoneal injection in mice bearing SC H460 NSCLC, HT29 colon carcinoma, Karpas 299 lymphoma, or MOLM-13 AML tumors. Treatment was initiated when tumors reached 100 mm³ in volume. sFLT01 (25 mg/kg) resulted in approximately 70% reduction in tumor volume in the H460 NSCLC model when control tumors reached an average volume of ~1,500 mm³ on day 18 (*P* < 0.0001) and a tumor growth delay of 42 days (Fig. 2A). sFLT01 (10 mg/kg) inhibited the growth of HT29 colon carcinoma tumors, resulting in tumors that were approximately 65% smaller in volume on day 39 when control tumors were an average volume of 1,000 mm³ (*P* < 0.0001) and a 38-day tumor growth delay (Fig. 2B). In the Karpas 299 lymphoma model, sFLT01 (25 mg/kg) reduced tumor volume by more than 75% on day 14 when the average volume of the control tumors was ~1,250 mm³ (*P* < 0.0001) and a 28-day tumor growth delay (Fig. 2C). sFLT01 (25 mg/kg) also inhibited the growth of MOLM-13 AML tumors, resulting in a 70% reduction in tumor volume on day 15 when control tumors were ~1,500 mm³ (*P* < 0.0001) and a 7-day tumor growth delay (Fig. 2D). There was no body weight loss in the mice treated with sFLT01 (data not shown).

RCC is a disease characterized by pathologic overexpression of VEGF and is a relevant clinical indication for therapeutics that interfere with the VEGF pathway (30). The antitumor and antiangiogenic activities of sFLT01 were shown in several RCC models including the 786-O xenograft and RENCA syngeneic SC and orthotopic models. sFLT01 (25 mg/kg) slowed the growth of 786-O RCC tumors, resulting in a 60% reduction in tumor volume on day 33 when control tumors were ~1,000 mm³ and generating a 20-day tumor growth delay (*P* < 0.0001; Fig. 3A). An anti-CD31 antibody was applied to 786-O tumor sections to visualize tumor endothelium, using immunohistochemistry (IHC) methods. Blood vessels in the vehicle-treated tumors that were collected from the control mice were mature and...
well-developed (Fig. 3B). In comparison, the blood vessels in the tumors from mice treated with 4 doses of sFLT01 and collected 1 day later were fragmented, lacked lumen formation, and were notably smaller (Fig. 3C). Morphometric analysis revealed that although the MVD was not reduced by sFLT01 treatment due to an increase in smaller, fragmented vessels, vessel and vascular areas, and lumen size and vessel perimeter were reduced, although the difference was not statistically significant ($P > 0.05$; Fig. 3D).

In addition to hVEGF-A and hPlGF, sFLT01 binds to both mVEGF-A and mPlGF (Fig. 1A and B); therefore, in addition to the 786-O xenograft model, the activity of sFLT01 could be shown in syngeneic models of RCC. In the SC RENCA tumor model, sFLT01 at 5 or 25 mg/kg inhibited tumor growth and reduced tumor volume by approximately 65% and 80%, respectively, on day 30 when control tumors were 1,500 mm$^3$ in volume ($P < 0.0001$; Fig. 4A). A 14- and 25-day tumor growth delay was achieved following administration of 5 and 25 mg/kg sFLT01, respectively. sFLT01 was also efficacious following orthotopic implantation of RENCA cells into the subrenal capsule of the kidney. sFLT01 (25 mg/kg) significantly increased median survival from day 31 in the control group to day 50 ($P < 0.0001$; Fig. 4B).

The antiangiogenic activity of sFLT01 in the SC RENCA model was evident when tumors were collected 1 day after the third dose and the tumor ECs were identified with an anti-CD31 antibody by IHC. The tumors in the control group were densely populated with blood vessels (Fig. 5A). In contrast, the tumors from mice treated with sFLT01 (5 mg/kg) contained fewer blood vessels throughout (Fig. 5A). Morphometric analysis (Fig. 5B) confirmed a significant reduction in MVD and lumen size ($P = 0.0014$ and 0.0050, respectively) but not in vessel perimeter or vascular and vessel areas ($P = 0.2266$, 0.4752, and 0.3549, respectively). The reduction in MVD in the RENCA model but not the in the 786-O RCC model may be attributed to the timing of the collection of the tumors. At later time points, the control tumors in some preclinical tumor models may have fewer but larger and more mature blood vessels (unpublished data). To investigate the effect of sFLT01 on the cellular components of tumor vasculature, antibodies against CD31 and NG2 were used to selectively identify tumor ECs and pericytes, respectively. Pericytes were found to be in close association with
tumor ECs in the control tumors (Fig. 5C). In the sFLT01 group, even though pericytes and ECs were present and in direct contact with each other (Fig. 5C), there were both fewer ECs and pericytes following treatment with sFLT01. Quantification of the pericytes that were identified by an anti-NG2 antibody in the RENCA tumors indicated a significant reduction in pericytes following treatment with sFLT01 (P = 0.0003; Fig. 5D).

To investigate the effect of sFLT01 in combination with chemotherapy, sFLT01 was administered either as a single agent or in combination with cyclophosphamide in the SC A673 Ewing’s sarcoma model. A lower dose of sFLT01 (5 mg/kg) alone resulted in an approximately 50% inhibition in tumor volume on day 17 when the control tumors reached ~1,000 mm³ and a tumor growth delay of only 4 days in this experiment (Fig. 6A). Once-weekly cyclophosphamide administration generated a tumor growth delay of 43 days. However, the combination regimen of cyclophosphamide plus sFLT01 was most effective, producing a tumor growth delay of 67 days determined when tumors reached ~1,000 mm³ in volume (P < 0.0001). There was no increase in body weight loss in the group treated with the combination regimen compared with cyclophosphamide alone (data not shown). Double immunofluorescence methods were applied to A673 tumors that were treated with sFLT01 alone, using an anti-human IgG1 antibody to detect sFLT01 and an anti-VEGFR2 antibody that is cross-reactive. sFLT01 was noticeably present in the necrotic areas of the tumor and was evident elsewhere in the tumor microenvironment (Fig. 6B). The detecting anti-human IgG1 antibody did not generate a signal in the control tumors (inset). VEGFR2 detected in the sFLT01-treated tumors was not specific to tumor endothelium and may be either present in the tumor microenvironment as soluble VEGFR2 or expressed by the A673.
Ewing’s sarcoma cells. This finding is not unlike the detection of the molecular expression of VEGF receptors in human Ewing’s sarcoma specimens (31).

To elucidate the changes that occurred in the tumor and tumor microenvironment following sFLT01 administration, gene expression profiling was conducted on A673 tumors collected 1 day after the third sFLT01 dose. Hierarchical clustering showed distinct molecular changes in the sFLT01-treated tumors compared with control tumors (Supplementary Fig. S1). Overall, the changes at the message level were modest. Correlation analysis of differential gene expression to other gene expression profile studies suggested activation of hypoxia-related pathways in the sFLT01-treated tumors (Supplementary Table S1). This result supports IHC data showing that sFLT01 exerts an antiangiogenic effect in vivo.

Discussion

sFLT01 has antiangiogenic activity due to its ability to bind to and neutralize VEGF-A and PIGF. It inhibited the proliferation of ECs and pericytes in vitro and resulted in both fewer ECs and pericytes in vivo in SC RENCA tumors. sFLT01 resulted in less vasculature and blood vessels that were fragmented, immature, and lacked well-developed lumens. The reduction in functional blood vessels by sFLT01 resulted in an inhibition in tumor growth and an increase in median survival in a wide variety of tumor types that represent NSCLC, colon carcinoma, RCC, Ewing’s sarcoma, lymphoma, and AML. The data presented here illustrate the robust efficacy of sFLT01 in preclinical studies. There were also no detectable side effects such as proteinuria, altered blood cell or serum chemistry profiles, or impairment of wound healing with sFLT01 (data not shown).

sFLT01 binds to mouse VEGF and PIGF and therefore can be evaluated in preclinical tumor models without the addition of a second agent directed toward the murine homologues. Therefore, direct comparisons of the efficacy or safety between bevacizumab and sFLT01 in standard xenograft models would not be equitable since bevacizumab does not bind murine VEGF and...
tumor growth may be driven primarily by mouse angiogenic factors rather than by human angiogenic factors produced by the malignant cells (32).

Preclinical studies have value in gauging the efficacy of antiangiogenic agents but may not necessarily predict toxicities that could be experienced in the clinic in combination therapies. For example, RCC patients experienced unacceptable side effects from a combination of bevacizumab and sunitinib that resulted in the discontinuation of a clinical trial (33). Differences between the human and mouse species exist with regard to pharmacokinetic and pharmacodynamic parameters, immune-mediated responses, and the tumor microenvironment. These dissimilarities can hinder the ability to fully translate the results generated in preclinical models to the clinical setting.

While the therapeutic benefit of interfering with the VEGF pathways has been robust in preclinical tumor models and validated in the clinic, the effect is modest and additional research is emerging which suggests that targeting PlGF may have value. PlGF overexpression in numerous human specimens of a variety of carcinomas versus normal counterpart tissues has been well documented (9–15). The contribution of PlGF to tumor angiogenesis was shown in tumor-bearing wild-type or Pgf−/− mice (34). Mice deficient in PlGF developed tumors that were smaller and developed less vasculature than tumors in wild-type mice. The same study revealed a synergy between PlGF and VEGF and a role for modulation of VEGF through the activation of VEGFR1 by PlGF. Hence, neutralizing PlGF in addition to VEGF at the onset may be more effective than a single target approach. However, this strategy is not without controversy, as conflicting reports exist on the basis of results generated in preclinical studies (35, 36). The value of a combinatorial approach against VEGF and PlGF will ultimately need to be determined in a clinical setting in a heterogeneous patient population that receives not only antiangiogenic therapy but also other modalities of treatment such as radiation and chemotherapy.

Alternatively, a drug such as sFLT01 may hold value as a second-line antiangiogenic agent in consideration of clinical data which suggest that PlGF may play a role in resistance to antiangiogenic therapies and tumor escape. Serum PlGF levels increased in colorectal cancer patients following bevacizumab therapy (22, 23). Similar observations have been made in patients with metastatic RCC who no longer responded to bevacizumab or in patients with prostate cancer who were treated with sunitinib (24, 25). The administration of sFLT01 to patients with elevated serum PlGF levels could offer a novel approach to neutralize PlGF that may be contributing to drug resistance and tumor escape. Since other factors may contribute to antiangiogenic resistance (37, 38), patients would be prescreened prior to sFLT01 therapy to identify the subpopulation of patients who would most likely respond. As a soluble decoy receptor, sFLT01 would neutralize PlGF independent of its source, that is, malignant cells and stroma or host tissues (19).

Some antiangiogenic drugs such as bevacizumab provide little benefit as a monotherapy and are delivered in combination with other treatments as standard of care (11, 39). While sFLT01 alone was efficacious in the A673 sarcoma model, sFLT01 generated a greater benefit in combination therapy with cyclophosphamide than with cyclophosphamide alone. This result suggests...
that an improved outcome could be achieved in the clinic with the incorporation of sFLT01 into a chemotherapy regimen. In addition, sFLT01 as a protein may be less toxic in combination with cytotoxic therapeutics than with the more promiscuous small molecule tyrosine kinase inhibitors (40). With the advent of targeted therapies, new approaches are combining antiangiogenic drugs with agents that selectively target pathways rather than with broad cytotoxic agents (41). Thus, the antiangiogenic properties of sFLT01 could be another useful combination strategy for oncology treatments.

With pathologic overexpression of VEGF and PIGF implicated in other diseases including age-related macular degeneration, diabetic retinopathy, and inflammatory disorders (32, 34, 42-45), the potential value of sFLT01 as an antiangiogenic drug extends beyond cancer therapy. Indeed, bevacizumab, which is approved for the treatment of certain cancers, is being investigated to treat neovascular macular degeneration (46). sFLT01 may offer...
a greater benefit in patients in whom PIGF in addition to VEGF is involved in the pathogenesis of this disease and other vascular disorders, thereby expanding its utility in the clinic.

Several drugs have been developed that either directly target PIGF or bind to the receptor VEGFR1 as agonists. Multitargeted tyrosine kinase inhibitors such as sorafenib and sunitinib are Food and Drug Administration-approved but do not selectively interfere solely with the PIGF pathways, as they also have activity against the PDGF, VEGFR2, Flt3, and c-kit pathways (11). TB-403 is a monoclonal antibody against PIGF and is currently in phase I clinical trials (26, 47). Aflibercept that most closely resembles sFLT01 in

Figure 6. sFLT01 in the human A673 sarcoma tumor model. A, sFLT01 (5 mg/kg, i.p., 2 times per week) inhibited the growth of s.c. A673 tumors, resulting in only a 4-day tumor growth delay when control tumors were ≈1,000 mm³ but a decrease in tumor volume by ~50% on day 17. sFLT01 enhanced the antitumor activity of cyclophosphamide (125 mg/kg, i.p., 1 time per week) by increasing the tumor growth delay of cyclophosphamide from 43 to 67 days when tumors reached a volume of ~1,000 mm³ in combination therapy. (P < 0.0001). B, an anti-human IgG antibody identified sFLT01 (green) in necrotic and vascular areas of A673 tumors (left). Inset, control tumor. VEGFR2 expression (red) by A673 cells was revealed with an antibody against human VEGFR2. Magnification ×4; scale bars, 250 μm. sFLT01 was present in areas of tumor vasculature (right). Magnification ×10; scale bar, 100 μm.
structure is in phase III trials for several cancer indications (48). Although it is not expected that an antiangiogenic drug will be fully efficacious as a stand-alone therapy, sFLT01 is a novel, next generation antiangiogenic agent with ability to bind to both VEGF and PlGF with a potentially better toxicity profile by not interfering with other signaling pathways. The results presented here show the robust efficacy of sFLT01 in both solid and hematologic cancers, thereby offering sFLT01 as promising antiangiogenic agent.

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Disclosure of Potential Conflicts of Interest

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