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Inhibition of Sphingosine Phosphate Receptor 1 Signaling Enhances the Efficacy of VEGF Receptor Inhibition

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Abstract

Inhibition of vascular endothelial growth factor receptor (VEGFR) signaling is an effective treatment for renal cell carcinoma but resistance continues to be a major problem. Recently, the sphingosine phosphate (S1P) signaling pathway has been implicated in tumor growth, angiogenesis and resistance to antiangiogenic therapy. S1P is a bioactive lipid that serves an essential role in developmental and pathological angiogenesis via activation of the S1P receptor 1 (S1P1). S1P1 signaling counteracts VEGF signaling and is required for vascular stabilization.

We used in vivo and in vitro angiogenesis models including a post-natal retinal angiogenesis model and a renal cell carcinoma murine tumor model to test whether simultaneous inhibition of S1P1 and VEGF leads to improved angiogenic inhibition.

Here we show that inhibition of S1P signaling reduces the endothelial cell barrier and leads to excessive angiogenic sprouting. Simultaneous inhibition of S1P and VEGF signaling further-disrupts the tumor vascular beds, decreases tumor volume, and increases tumor cell death compared to monotherapies.

These studies suggest that inhibition of angiogenesis at two stages of the multi-step process may maximize the effects of antiangiogenic therapy. Together these data suggest that combination S1P1 and VEGFR targeted therapy may be a useful therapeutic strategy for the treatment of renal cell carcinoma and other tumor types.

Keywords

Renal cancer; VEGF; S1P; angiogenesis; tumor

Conflicts of Interest: BF, RA, DB, GE, JS, TW, PH, JM, MU, SC, DG, and LB: employees of Eli Lilly.

Introduction:

Vascular Endothelial Growth Factor A (VEGF) is the predominant growth factor expressed by tumor cells to drive angiogenesis and solid tumor growth. Antiangiogenesis therapies targeting VEGF or its receptor VEGF receptor 2 (VEGFR) and immune therapies have been clinically demonstrated to be effective in prolonging overall survival and progression-free survival while significantly improving the quality of life for certain cancer patients [1–6]. In tumors such as clear cell renal cell carcinoma (RCC), where VEGF pathway inhibition has demonstrated single agent activity, there are five approved agents that target VEGF signaling. Among these are four VEGFR tyrosine kinase inhibitors; sunitinib, sorafenib, axitinib and pazopanib [4–6]. Unfortunately not all patients benefit from these VEGF pathway inhibitors. Some patients do not respond to this class of inhibitors, some ultimately develop resistance, and complete responses are extremely rare. For this reason there is an urgent need to identify new therapeutic approaches to inhibit tumor angiogenesis with mechanisms of action that are distinct from and/or may complement VEGF/VEGFR modulators. Combinations with other vascular pathway modulators such as sphingosine-1-phosphate (S1P) inhibitors may fill a gap and enable vascular targeting in otherwise VEGF-pathway independent blood vessels.

S1P is a bioactive lipid and important regulator of vascular function and immune cell trafficking [7]. S1P has also been shown to be a potent inducer of many of the hallmarks of cancer including tumor angiogenesis, cancer cell growth, immune modulation, migration and invasion [8, 9]. S1P signaling is mediated via five G-protein coupled endothelial differentiation receptors (S1P_{1–5} receptors). S1P signaling is diverse and involves many signaling pathways known to be important in cancer including the PI3K, MAPK and pSTAT3 pathways [8]. The S1P receptor 1 (S1P₁), in particular, has been shown to play a key role in angiogenesis, which was first demonstrated by S1P₁ genetic deletion studies in mice [11]. Loss of S1P₁ function results in embryonic lethality due to severe hemorrhage likely associated with defects in pericyte recruitment and vessel maturation. More recent studies evaluating endothelial specific S1P₁ deletion indicate S1P₁ signaling also inhibits angiogenic sprouting in the retina of postnatal mice [12–14]. S1P signaling via S1P₁ appears to be part of a negative feedback mechanism that is required for maintaining blood vessel integrity by counteracting VEGF signaling and excessive angiogenic sprouting [13]. Our current understanding of S1P signaling in the vasculature indicates that S1P₁ plays a critical role in limiting VEGF dependent angiogenesis and promoting vascular stability via enhancement of endothelial cell-cell junctions. Loss of S1P₁ function has an opposite effect leading to VEGF dependent hypersprouting angiogenesis, increased vascular permeability and loss of vascular function [12–14]. S1P₁ inhibition leads to disorganized and non-functional angiogenesis in non-proliferating tumor vessels where VEGF inhibition was not previously effective. The blood vessels resulting from S1P₁ antagonism are fragile and effectively eliminated by blockade of VEGF signaling.

Preclinical studies have shown that modulation of S1P₁, using several different approaches, will inhibit angiogenesis and tumor growth. FTY720, a well characterized agonist that activates S1P_{1,3,4} and 5, significantly decreases tumor angiogenesis as well as vascular permeability and tumor cell viability [15]. The combination of FTY720 with a VEGFR

kinase inhibitor was shown to be additive, suggesting the potential for improving VEGF pathway directed therapies. A monoclonal antibody specific for S1P (S1P mAb) also significantly inhibited tumor angiogenesis and growth in several animal models of human cancer [16–18]. These effects were associated with inhibition of S1P induced cancer cell proliferation and release of pro-angiogenic factors. These inhibitors did not inhibit S1P1 specifically, In fact, FTY720 behaves as a functional antagonist and initially activates the S1P1 receptor followed by the internalization and degradation of the receptor. FTY720 is not selective for S1P1 and also inhibits S1P3–5 signaling. Thus, selective S1P1 inhibitors may provide more attractive targets due to their specificity. Selective S1P1 inhibitors described in the literature also disrupt the tumor vasculature and inhibit tumor growth in pre-clinical xenograft tumor models but to our knowledge have never been tested in combination with VEGFR inhibition [19, 20]. We have previously shown that tumors pretreated with a VEGFR tyrosine kinase inhibitor (TKI) upregulate many hypoxia regulated factors including sphingosine kinase 1 (SPHK1) [18]. SPHK1 catalyzes the production of S1P and it is also expressed in many tumor types including RCC [8]. S1P neutralization was able to slow tumor growth in treatment naïve as well as VEGFR TKI resistant tumors [18]. Together, these studies suggest modulation of vascular VEGF/VEGFR and S1P1 signaling may provide a novel therapeutic combination approach for inhibiting tumor angiogenesis and tumor growth. Here we explore the mechanism of S1P1 inhibition. We show inhibition of S1P1 signaling destabilizes endothelial cell junctions, delays vessel maturation, and promotes vessel sprouting in response to VEGF. These effects render the tumor vasculature more sensitive to VEGFR inhibition leading to greater antiangiogenic and antitumor activities.

Material and Methods:

Endothelial cell barrier assays.

To assess endothelial barrier function, VE cadherin staining of an endothelial cell monolayer and an endothelial cell permeability assay were used. For both assays, adult human dermal microvascular endothelial cells (HMVECs; Lonza, Walkersville, MD) were grown in EGM2-MV on collagen I coated flasks prior to assay. For VE cadherin staining, HMVEC monolayers were established in 96-well fibronectin-coated plates by plating 42,000 cells in 100 μ L of EGM2-MV media and incubated for 3 days. Prior to the day of compound addition, fresh media was added to the cells. Cells were treated overnight (~18 hours) with 3nM Ex82 after which the cells were fixed with 3% paraformaldehyde for 10 mins. Cells were washed, permeabilized with PBS + 1% BSA + 0.5% Triton X100, and stained for VE cadherin using a goat anti-VE cadherin antibody (BD Biosciences #555661, Franklin Lakes, NJ) at 1:50 and with Hoechst 33342 (Invitrogen, 1:1,000) followed by secondary antibodies (goat anti-mouse Alexa Fluor-488; Invitrogen) at 1:400. Cells were imaged with a Cellinsight NXT imager using a 20x objective (Thermo Scientific, Tewksbury, MA). For the permeability assay, HMVEC monolayers were established on 1 μ m pore transwells (Corning, Corning, NY) coated with 5 μ g/mL fibronectin (Life Technologies, Carlsbad, CA) by plating 50,000 cells in 100 μ L of EGM2-MV media [21]. Media was added to the bottom of the transwell and incubated for 3 days. The day prior to addition of drugs, fresh media was added to the transwell and receiver plates. HMVEC monolayers were treated overnight (~18

hours) with a dose response of Ex82. The following day 1.8 mg/mL of FITC-dextran (MW 40,000; Sigma, St Louis, MO) was added to the transwell and incubated for 3 hours. Fluorescence within the receiver plate was measured on a fluorescent plate reader (excitation 380, emission 505). To ensure that the changes in permeability were due to effects on the barrier function and not loss of cell number or viability; at the end of the experiment, Presto Blue (Life Technologies, Carlsbad, CA) was added to the transwell plate for 1 hour and read with a plate reader (excitation 380, emission 505).

In vitro S1P1 inhibitor assay

A S1P1 beta-arrestin recruitment assay was used to characterize the in vitro inhibition of S1P1. We used the S1P1 expressing cells and PathHunter detection kit (DiscoverRx Corporation, Fremont, CA) to measure inhibition of β -arrestin recruitment to S1P1 by S1P (Avanti Polar Lipids, Alabaster, AL). Briefly, cells were plated overnight at 37°C and 5% CO₂ in OPTI-MEM + 10% FBS (Invitrogen, Grand Island, NY). Appropriate dilutions of inhibitor compounds were added to the cells, incubated for 30 minutes at 37°C followed by addition of an EC80 of S1P for another 90 minutes at 37°C. The plate was allowed to equilibrate at room temperature for 30 minutes before adding detection reagent and incubating 60 minutes at room temperature. Luminescence was measured and quantified using an appropriate reader.

In vivo target inhibition of murine phosphorylated VEGFR2.

Protocols essentially described by T. Burkholder et. al. were used to assess VEGFR2 inhibition in vivo [23]. Briefly, female athymic nude mice (22 g) were treated orally with compounds for 2 hours (Ex82: 30 mpk, sunitinib: 20 mpk) or 24 hours (DC101: 20 mpk) before VEGFR was stimulated by tail vein injection with murine VEGF (500ng, Peprotech 450–30). Lungs were collected 5 minutes after VEGF stimulation and homogenized in Tris lysis buffer (MSD R60TX-3) containing MSD's protease/phosphatase inhibitor pack (MSD R70AA-1). Western blot analysis of lung lysates was performed to detect and measure VEGFR activation via phosphorylated VEGFR (pVEGFR). Antibodies from Cell Signaling were used: pVEGFR: 2478, B-Actin: 4967, and total VEGFR2:2479. Blots were developed using Pierce's chemiluminescent Supersignal West Pico and Femto substrates. Bands were visualized using the Fujifilm LAS4000 and quantified using the software Image J. pVEGFR was normalized using B-actin, averaged, and compared to the mean of the vehicle group to obtain the fold change. Statistics were performed using JMP software. Data are representative of two studies (n=8 animals total).

Multiplexed immunohistochemistry analysis of tumors.

Multiplexed fluorescent immunohistochemistry and high-content tissue imaging and quantification were performed as described previously [24]. For the angiogenesis panel, blood vessels were examined with CD34 (Biolegend, 1:100, San Diego, CA) and S1P1 (Santa Cruz, 1:100, Dallas, TX) antibodies multiplexed with a myofibroblast/pericyte marker (Cy3- conjugated smooth muscle actin, SMA, Sigma, 1:400, St Louis, MO). Secondary antibodies conjugated to Alexa Fluor-488 or -647 anti-rat or anti-rabbit were used for detection. For the tumor health panel, blood vessels (CD34), proliferation (Ki67, NeoMarkers, 1:100), and apoptosis (TUNEL, Roche, Indianapolis, IN) were examined as

described elsewhere [24]. Whole tumor sections were imaged and quantified using the iCys research imaging cytometer. The percent of each marker normalized to the total tumor area identified with Hoechst 33342 (Invitrogen, 1:1,000, Grand Island, NY) was determined. Differences between treatment groups were assessed using ANOVA analysis with SAS JMP software (Cary, NC).

Retina whole mount assay.

Following daily intraperitoneal injections of 30 mg/kg Ex82 from postnatal day 2 – 5, or 20 mg/kg anti-VEGFR (DC101) at day 2 and day 4, neonatal mice (female and male in C57BL/6 background) were sacrificed at postnatal day 5 and eyes collected into formalin. Retinas were dissected and blocked in PBS, 0.2% Triton X-100 and 10% goat serum overnight at 4 °C, and then incubated in blocking solution successively overnight in isolectin GS-IB4, Alexa Fluor 647 (Invitrogen, Grand Island, NY) or primary antibodies (CD31, MEC13.3, BD Pharmingen (Franklin Lakes, NJ); collagen IV, Abcam (Cambridge, MA); NG2, Millipore; Ter 119, BD Pharmingen (Franklin Lakes, NJ), and secondary antibodies (Jackson, West Grove, PA) each diluted 1:200 in blocking solution. Retinas were washed (four to five times for 1 h) in PBS, flattened and photographed using a Nikon Ti microscope. Vascular progression (assessed by measuring the distance from the center of the retina to the angiogenic front of the retina), number of tip cells and vascular density of the remodeling plexus were quantified with anti-CD31 staining using FIJI software. Results were presented as means $\pm$ SEM. Statistical significance of all data was analyzed using one way ANOVA (Dunnett's test) in GraphPad Prism 6 software (La Jolla, CA). P-values <0.05 were considered to be statistically significant. Representative images were acquired using confocal Nikon A1 microscope.

Tumor xenograft assay.

For subcutaneous xenograft RCC tumor models, female nude beige mice (Charles River Laboratories, MA), 6–8 weeks of age were used. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Beth Israel Deaconess Medical Center. 786-O RCC cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). 786-O cells were grown in RPMI 1640 medium (ATCC, Manassas, VA). The medium was supplemented with 2mM L-glutamine, 10% fetal calf serum and 1% streptomycin (50 μ g/ml) and cells were cultured at 37°C with 5% CO₂. RCC cells were harvested from subconfluent cultures by a brief exposure to 0.05% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed once in serum-free medium and resuspended in PBS. Only suspensions consisting of single cells with greater than 90% viability were used for the injections.

Cells were injected subcutaneously unilaterally (1×10^7 cells) into the flanks of the mice. Vehicle, sunitinib (50 mg/kg daily by gavage), S1P1 antagonist Ex82 (30mg/kg daily by gavage), or the combination of sunitinib and S1P1 antagonist Ex82 begun when the tumors reached a diameter of 12 mm as per our previous reports [25, 26]. Tumors were measured daily during therapy to generate tumor growth curves. The delta t/c or D[T/C] was calculated using $D[T/C] = 100 * (\text{Treated Tumor Volume} - \text{Baseline Tumor Volume}) / (\text{Control Tumor Volume} - \text{Baseline Tumor Volume})$. The scale on D[T/C] normally runs between 0 and 100.

100 means the treated tumor volume is no difference from vehicle. 0 means that the treated tumor volume is the same as baseline or stasis.

Arterial Spin Labeled MRI

Imaging of tumor blood flow was performed using arterial spin-labeled magnetic resonance imaging (MRI;ASL MRI) as previously described [25]. Briefly mice were anaesthetized, and placed in the supine position on a 3 cm in diameter custom-built surface coil. Images were acquired using a 3.0 T whole-body clinical MRI scanner (3T HD; GE Healthcare Technologies, Waukesha, WI). A single slice ASL image was obtained with a single-shot fast spin echo sequence (SSFSE) using a background-suppressed, flow-sensitive alternating inversion-recovery strategy. The single transverse slice of ASL was carefully positioned at the center of the tumor, which was marked on the skin with a permanent marker pen for follow-up MRI studies. To determine tumor blood flow, a region of interest was drawn freehand around the peripheral margin of the tumor by using an electronic cursor on a T2-weighted anatomical reference image that was then copied to the ASL image. The mean blood flow for the tumor tissue within the region of interest was derived. For display, a 16-color table was applied in 10 mL/100 g/min increments ranging from 0 to 160 mL/100 g/min, with flow values represented as varying shades of black, blue, green, yellow, red, and purple, in order of blood flow. Tumor blood flow was analyzed with repeated measures ANOVA following the previously described procedure [27].

Reagents:

The S1P1 inhibitor tool compound, Ex 82 [28], and DC101 were prepared and provided by Eli Lilly and Company. Sunitinib was purchased from LC Laboratories (Woburn, MA).

Results:

S1P1 small molecule inhibitor Ex82 potently inhibits S1P1 activity and endothelial barrier function

To study the effects of S1P1 inhibition on endothelial function, we first identified an S1P1 small molecule tool compound from the patent literature [28, 29] (Ex82 in WO2010072712). Compounds from this scaffold have been reported to be potent, selective S1P1 inhibitors with pharmacokinetic (PK) properties that allow for long lasting in vivo target modulation as measured by peripheral lymphocyte depletion in mice following oral dosing [30]. Our characterization of Ex82 showed it to potently inhibit S1P dependent activation of S1P1, in the 5 nM range, with selectivity for both the human and mouse receptors (Table 1). Ex82 was inactive against the other S1P receptors, S1P2, S1P3, S1P4 and S1P5 in both the agonist (activation of S1P1) and antagonist (inhibition of S1P1) modes, which allows for investigating S1P1 dependent biology (Table 1). These results were in agreement with published data on compounds from this chemical series [19, 30]. Importantly, Ex82 is a selective inhibitor of S1P1 that does not directly activate the receptor or induce receptor internalization and degradation like other reported S1P1 agonists. This allows for the investigation of direct S1P1 inhibition in vitro and in vivo.

We next evaluated the effects of S1P1 inhibition by Ex82 on endothelial function. Since S1P1 has been shown to play an essential role in vascular integrity and barrier function [31], we determined if inhibition of S1P1 would disrupt endothelial cell junctions and increase permeability of an endothelial monolayer. Staining of a HMVEC monolayer with the endothelial junction protein VE-Cadherin showed that while HMVECs formed tight cell junctions with a thick layer of VE-Cadherin staining (Figure 1A, arrow), treatment with Ex82 weakened endothelial junctions as shown by decreased thickness with discontinuous VE-cadherin staining between cells (Figure 1B and C arrows). The loss of barrier function with Ex82 was confirmed by using a transwell permeability assay which measures the passage of FITC-labeled dextran across an endothelial monolayer [21]. Ex82 increased the permeability to FITC-dextran in a dose dependent manner with an IC₅₀ of 16.0 nM (Figure 1D). We further characterized the effect of S1P and Ex82 using a transendothelial electrical impedance assay [31, 32]. This assay measures changes in electrical impedance relative to a voltage applied to a monolayer of endothelial cells [32] and is useful for assessing the modulation of endothelial barrier function by S1P1 and S1P. S1P treatment (10 nM) of an endothelial monolayer strongly increases electrical impedance whereas Ex82 has the opposite effect and significantly decreases electrical impedance (Supplemental Figure S1). These results are consistent with the known barrier function of S1P1. In addition, pretreatment with Ex82 blocked the S1P dependent increase in electrical impedance (Supplemental Figure S1). All of these results demonstrate that Ex82 is a potent and specific S1P1 inhibitor with endothelial barrier disrupting properties consistent with the expected effect of S1P1 inhibition in endothelial cells.

S1P1 inhibitor Ex82 modulates circulation of mouse peripheral lymphocytes.

To determine the potential for using Ex82 in vivo, we assessed the effect of Ex82 on circulating mouse lymphocytes, a well-validated assay for characterizing the in vivo effects of S1P1 inhibition [33, 34]. Oral dosing of Ex82 induced a rapid and dose-dependent reduction in circulating mouse lymphocytes at 4 hours post dose (Figure 1E). At this 4 hour timepoint maximal reduction in circulating lymphocytes was observed at doses of 3 mg/kg (mpk) or greater. At 24 hours post-dose, 30 mpk of Ex82 reduced circulating lymphocytes by greater than 85% compared to vehicle control and there was sufficient plasma exposure of Ex82 to ensure robust S1P1 inhibition based on a IC₅₀ for S1P1 inhibition of 4 nM (Table 1). It was for these reasons a 30 mpk once a day dose of Ex82 was used for all subsequent in vivo mouse studies.

S1P1 inhibition enhances VEGF activation of VEGFR2.

S1P dependent activation of S1P1 has been shown to inhibit VEGF activation of VEGFR2 and sprouting angiogenesis [13]. For this reason, we hypothesized potent inhibition of S1P1 with Ex82 would enhance VEGF dependent activation of VEGFR2 and this would have the potential to improve response to VEGFR targeted agents. To test this hypothesis, we investigated the effects of S1P1 inhibition on VEGF activation of VEGFR2 in vivo. Tail vein injection of murine VEGF strongly activated VEGFR2 (Figure 2). Pretreatment with Ex82, at a dose that potently inhibits S1P1 in vivo, prior to VEGF tail vein injection resulted in a significant increase in pVEGFR2 compared to VEGF alone (Figure 2). We next use DC101, a monoclonal antibody that blocks murine VEGFR2, as a tool compound to determine the

combined effects of S1P1 and VEGFR2 inhibition. Since the standard of care for VEGFR inhibition in metastatic RCC patients is a VEGFR tyrosine kinase inhibitor we also used sunitinib for our in vivo proof of concept studies. Pretreatment with the Anti-VEGFR2 antibody DC101 or the VEGFR2 kinase inhibitor sunitinib potently inhibited the VEGF dependent activation of VEGFR2 (Figure 2). Together, these results demonstrate rationale for co-inhibition of S1P1 and VEGFR2..

Co-targeting S1P1 and VEGFR2 pathways induced vascular regression.

To evaluate the antiangiogenic impact of targeting S1P1 and VEGFR2 pathways in vivo, we used the well-established mouse retinal angiogenesis model. Previous studies showed that genetic ablation of S1P1 receptor in retinal blood endothelial cells induced hypersprouting and disorganization of the remodeling plexus with retained perivascular cell coverage [13]. In agreement with this study, inhibition of S1P1 by Ex82 increased endothelial tip cells at the angiogenic front and vascular density of the remodeling plexus (Figure 3 A-B). This disorganized angiogenic process, however, decreased the progression of blood vessels from the optic disc (OD) into the avascular retinal tissue (Figure 3 A-B). Despite the presence of pericytes, we also observed hemorrhage within the plexus, which is consistent with endothelial barrier destabilization and increased permeability (Supplemental Figure 2 A-B).

Previous studies have shown that targeting VEGFR2 with a selective antibody (DC101) elicits a potent antiangiogenic effect on the retinal vasculature [35]. To explore the benefit of targeting both VEGFR2 and S1P1, we used 20mg/kg on days 2 and 4 of DC101 with 30mg/kg Ex82 daily. This dose of DC101 is below the dose needed to see maximal effects in a developing retina and is permissive to see additive or combination effects. The combination of VEGFR2 and S1P1 inhibition in the retina triggered a significant regression of CD31-positive endothelial cells in the remodeling plexus, leaving behind basement membrane sleeves of collagen IV (Figure 3A - white arrowheads). This resulted in a lower vascular density in the combination treatment compared to the vehicle treated mice. The combination also decreased the vascular progression and increased the number of endothelial tip cells compared to VEGF inhibition alone (Figure 3A-B). With this combination, the areas of hemorrhage were reduced within the vascular plexus and restricted only to the angiogenic front indicating that the remaining vessels in the remodeled area are less permeable (Supplemental Figure 2). These results suggest increased sensitivity of remodeling blood vessels to anti-VEGFR2 therapies when the vessels are destabilized by S1P1 inhibition. These data, along with our mouse lung data, support the idea of combining VEGF and S1P1 pathway inhibition as a novel antiangiogenic therapeutic regimen that may improve upon VEGF pathway blockade alone.

Combination S1P1 and VEGF pathway inhibition decreased RCC tumor growth and blood flow.

RCC is a vascular tumor that is highly dependent on VEGF likely due to the VHL loss seen in most RCC. In RCC, VEGF pathway inhibition has shown clinical effects, and we have previously shown that VEGFR TKI therapy leads to induction of the S1P pathway [25]. Since inhibition of S1P1 signaling by Ex82 destabilizes endothelial cell junctions, delays vessel maturation, and promotes vessel sprouting in response to VEGF [13] (Figure 1 and 3),

we hypothesized that these vascular features following S1P1 inhibition would render the tumor vasculature more sensitive to VEGF pathway blockade. To test this hypothesis we evaluated the effect of S1P1 inhibition alone and in combination with sunitinib in the 786-O VHL deficient RCC murine xenograft model compared with a vehicle control group, (n=6–8 per group). Treatment with either sunitinib or Ex82 led to slowed tumor growth as single agents compared to vehicle control (Figure 4) (P=0.0082 for Ex82 vs vehicle and <0.0001 for sunitinib vs vehicle). The addition of Ex82 to sunitinib led to improved tumor growth control compared to sunitinib alone (P=0.008). When dosed in mice alone or in combination with sunitinib, Ex82 treatment did not lead to weight loss or other toxicity as assessed by activity level.

We have previously shown that VEGFR TKI treatment reduces tumor blood flow measured by ASL MRI by 50–80% at 3 days after treatment from baseline [36, 37]. In this model, analysis of tumor blood flow data showed that there was no difference in baseline blood flow at day 0, but after 7 days of treatment, the S1P1 antagonist Ex82 resulted in lower tumor blood flow compared to vehicle treated tumors (Figure 5 A,B, D; p = 0.0397). Mice treated for longer periods of time were imaged in the last week of their treatment depending on availability of the scanner. At later time points (day 26 +/- 4 days), tumors from mice treated with sunitinib and Ex82 showed lower tumor blood flow than vehicle (P values shown in Figure 5 E), and there was a trend toward lower tumor blood flow in the combination arm compared to the sunitinib alone arms (p=0.1043).

Sunitinib and S1P1 inhibitor combination lead to apoptosis of tumor cells in vivo.

Multiplexed immunohistochemistry panels to assay tumor angiogenesis and health (proliferation and apoptosis) were used to further study the mechanism of the combined effect of sunitinib and Ex82. The percent area of tumor vessels (labeled with CD34) tended to increase with S1P1 inhibition (20.4% for vehicle versus 30.5% with Ex82; Figure 6A-iii and 6A-v), consistent with the observed increased EC sprouting seen in the mouse retinal assay (Figure 3). As expected, sunitinib significantly reduced the percent area of vessels (6A-ii), but combination of Ex82 with sunitinib did not lead to significant further reduction in tumor vessels (7.2% for sunitinib and 4.8% for the combination; Figure 6A-iv, and 6A-v). S1P1 expression increased with Ex82 (p=0.0073) and this was significantly reduced with sunitinib and the combination treatment (p=0.0027 Ex82 vs. sunitinib and p=0.0052 Ex82 vs. combination; Figure 6A-iii and 6A-vi). Examination of the effects of treatment on tumor cell proliferation showed little effect of any of the treatments (percent area of Ki67; Figure 6B, 6B-v). Sunitinib treatment alone led to a non-significant increase in the percent area of TUNEL positive cells compared to vehicle (Figure 6B-ii and 6B-vi and 6C), while Ex82 single treatment had no effect on tumor cell apoptosis (Figure 6B-iii and 6B-vi). The combination treatment, however, significantly increased TUNEL staining more than the vehicle or either of the single agents. The percent area of TUNEL was 25.3% for the combination, compared to 4.9% for the vehicle (p=0.0003), 10.9% for sunitinib (p=0.0065), and 3.06% for Ex82 (p<0.0001; Figure 6B-vi). Since S1P1 was not expressed on tumor cells (Figure S3) these effects on tumor cell death are likely to be attributed to secondary effects due to the direct effects on the functional tumor vascular network, despite a modest reduction in vessel density.

Discussion:

Resistance to antiangiogenic therapy is a major obstacle in the management of metastatic RCC as well as other tumor types. Tumor angiogenesis is initiated and largely driven by VEGF, especially in RCC in which the VHL deficiency (including, mutation, deletion or loss of heterozygosity) renders the tumors highly dependent on VEGF [25, 38]. However, other angiogenic pathways are also required for the formation of functional tumor vascular beds [2, 25, 39]. A goal of our work has been to identify new endothelial specific targets that can be rationally combined with VEGFR targeted agents to improve their efficacy in inhibiting tumor angiogenesis. There is a well-characterized role for endothelial specific S1PR1 signaling in angiogenesis [11–13, 40] and well-known effects of tumor specific SPHK1-HIF axis associated with resistance to VEGFR TKI therapy [9, 16, 18, 41, 42]. Thus we investigated whether targeting endothelial S1P1 signaling with Ex82 a potent, selective inhibitor of S1P1, may combine with VEGFR inhibition to target angiogenesis at two main pathways leading to greater reduction in tumor growth.

The lack of S1P1 signaling has previously been shown to result in local tissue hypoxia which in turn enhanced VEGF production and VEGF dependent endothelial proliferation and sprouting angiogenesis [12–14]. Additionally, we have previously shown that VEGFR inhibition leads to induction of tumor hypoxia and induction of SPHK expression [18]. Based on these observations, we hypothesized that inhibition of VEGF signaling would modulate the tumor microenvironment to make blood vessels more dependent on S1P signaling and that S1P1 inhibition would enhance VEGF induced angiogenesis rendering resistant vessels sensitive to anti-VEGF therapy. Combined inhibition of VEGFR and S1P1 in this setting would result in enhanced inhibition of angiogenesis compared to either agent alone.

We show that inhibition of S1P1 alone destabilized the retinal vasculature resulting in hypersprouting blood vessels. The hypersprouting was accompanied by vascular hemorrhage as seen with TER 119 staining of red blood cells. Our results obtained with Ex82 phenocopied the results obtained by genetic knockout of endothelial S1P1 suggesting Ex82 modulates S1P1 dependent vascular biology in vivo [11–14]. When VEGF pathway inhibition was combined with S1P1 inhibition, there was a significant reduction in vascular progression and vascular density. Importantly, we saw evidence of empty basement membrane sleeves with the combination treatment, which indicates vascular regression [43]. These results uncover the dynamic sensitivity of remodeling blood vessels, which have been destabilized by S1P1 antagonism, to anti-VEGFR2 therapies. This indicates mechanistically that S1P1 inhibition makes the vessels more sensitive to VEGFR2 inhibition by making the vessels more dependent on VEGF signaling leading to reduced tumor growth and tumor cell apoptosis. These data support the development of this combination to enhance the sensitivity to VEGFR targeting.

We next evaluated the benefit of combined S1P1 and VEGFR2 therapy in a 786-O VHL deficient mouse xenograft model of RCC. We have previously shown that this model is dependent on SPHK1/S1P signaling when tumors progress on anti-VEGFR2 therapy [18]. In addition, the 786-O model exclusively expresses S1P1 on the endothelial cells of the tumor

associated vasculature. Serial ASL MRI perfusion imaging studies showed inhibition of S1P1 reduced tumor blood flow but not to the extent of sunitinib. The combination of S1P1 and VEGFR inhibition reduced blood flow even further. The magnitude of reduction in flow induced by sunitinib alone may mask the additional effects of S1P1 inhibition, although a trend for decreased blood flow was seen with the combination. Differences observed in tumor blood flow after S1P1 inhibition versus VEGFR inhibition further support the distinct effects of VEGFR2 and S1P1 on the tumor vasculature.

Interestingly, treatment with the combination but not either single agent alone led to a dramatic induction of tumor cell apoptosis demonstrating the potential increase in clinical response that this combination could have. The specific stresses placed upon the tumor cells with combination treatment are not fully known but may be in part due to increased tumor hypoxia and nutrient deprivation. We believe the significant effects on tumor apoptosis are secondary to the effects on the vasculature as S1P1 expression was not detected on tumor cells. S1P1 is well expressed in the tumor associated blood vessels in 786-O xenografts and in all other tumor xenograft models we have characterized. To date, there is only one known model (SK-Hep-1) which shows both tumor and tumor associated blood vessel S1P1 expression (Supplemental Figure 3).

It is also likely that the S1P pathway modulates the tumor immune microenvironment but the specifics of these effects are not yet fully understood and should be explored in an immune competent model. One known class effect of S1P inhibitors is their ability to modulate circulating lymphocytes. S1P has potent roles in limiting T cell egress from tissues into circulation and we also demonstrate that S1P1 inhibition with Ex82 reduces circulating lymphocytes. S1P1 signaling has been shown to drive Treg cell accumulation in tumors limiting CD8+ T cell recruitment and activation thus promoting tumor growth [44]. Testing of these agents in immune competent models may help elucidate the role of S1P inhibition in enhancing of immune mediated anti-tumor responses. Moreover, it will be important to assess the effects of S1P inhibition in combination with VEGF and PD1 pathway inhibition.

Inhibitors of the S1P pathways are currently being developed in the clinical setting. Inhibition of the S1P pathway has been achieved by two main strategies. S1P receptor modulators such as FTY720 mimic S1P and have been shown to have activity in multiple sclerosis, allograft rejection and inflammatory bowel disease [45]. An antibody against S1P (Sphingomab) has also been shown to have antitumor and antiangiogenic effects in preclinical models but did not meet its primary endpoints in a phase II clinical trial [18, 46].

In summary, using a potent and selective antagonist tool compound against endothelial protein S1P1 (Ex82), alone and in combination with VEGFR targeted agents, we show S1P1 inhibition destabilizes endothelial junctions in vitro and in vivo during the early and remodeling/maturation phases of retinal and tumor angiogenesis which leads to vascular beds that are vulnerable to VEGF pathway inhibition (Figure 7). S1P1 signaling is distinct yet complementary from the initiation phase of angiogenesis where VEGFA/VEGFR2 signaling is dominant. Targeting S1P1 and VEGFR2 simultaneously provides a novel therapeutic approach by inhibiting two mechanisms required for functional vasculature. Combined inhibition has the potential to enhance response rates compared to currently

approved anti-angiogenic agents and this combination has the potential to overcome S1P dependent resistance to anti-VEGF pathway therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation List:

VEGFR	Vascular endothelial growth factor receptor
S1P	Sphingosine phosphate
S1P1	S1P receptor 1
RCC	Renal cell carcinoma
SPHK1	Sphingosine kinase 1
TKI	VEGFR tyrosine kinase inhibitor
SPHK1	Sphingosine kinase 1

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Figure 1. S1P1 antagonist Ex82 disrupts endothelial barrier function and oral dosing reduces circulating mouse lymphocytes in a dose dependent manner.

Staining of a HMVEC monolayer with VE-Cadherin (green) and nuclear Hoechst 33342 (red) is shown after treatment with vehicle (A) or Ex82 (B and C). Junctions between endothelial cells show a thick area of VE-Cadherin staining with vehicle and thinning and disruption of junctions (white arrows) after Ex82 exposure. The loss of barrier function with Ex82 was confirmed by using a transwell assay which measures the permeability of FITC-labeled dextran across an endothelial monolayer. (D) Ex82 increased the permeability to FITC-dextran in a dose dependent manner with an $IC_{50} = 16.04$ nM. Oral dosing of Ex82 in mice led to a dose dependent reduction in circulating mouse lymphocytes at 4 hours post dose. Maximal reduction in circulating lymphocytes is shown at a dose of Ex82 at 3 mg/kg (mpk) or greater (E).

Figure 2. Inhibition of S1P1 enhances VEGF activation of endothelial VEGFR2.

Mice were orally dosed with compounds for 2 hours (Ex82: 30 mpk, Sunitinib: 20 mpk) or 24 hours (DC101: 20 mpk) followed by iv injection of murine VEGF to activate VEGFR. Lungs were collected 5 minutes after VEGF stimulation and western blot analysis of lung lysates was performed to detect and measure VEGFR activation. The S1P1 inhibitor Ex82 increases VEGFR activation 1.8 fold (p-value < 0.0001), while the VEGFR inhibitor DC101 decreases VEGFR activation by 53% (p-value < 0.0004). Sunitinib was used as a control and 95% target inhibition was achieved (p-value < 0.0001).

Figure 3. Co-targeting S1P1 and VEGFR2 pathways induces vascular regression.

Whole-mount staining of blood vessels by anti-CD31 (endothelial cell membrane) and anti-collagen IV (extracellular basement membrane) in mouse retinas of young pups (postnatal day 5) treated with vehicle or anti-VEGFR2 (DC101, 20 mpk) and S1P1 antagonist (Ex82, 30 mpk) or the combination of DC101 and Ex82 (A). Bottom panels represent a higher magnification of the retinal remodeling plexus (white boxes). Vessel regression was identified by collagen IV positive and CD31 negative structures (sleeves of former blood vessel basement membranes – white arrowheads). Quantification of vascular progression

from the retinal center (OD – optic disc) to the angiogenic front, endothelial tip cells at the angiogenic front and vascular density of the remodeling plexus (B). Results are pooled from 3 independent experiments (n = 6 animals per group per experiment, mean±s.e.m, one way ANOVA Dunnett's test). Scale bars: top panels = 200 μ m, bottom panels = 50 μ m.

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Treatment Group	N	Mean Tumor Volume (mm ³)	p-Value (Tumor)			D[T/C] %
			Vehicle	EX82	Sunitinib	
Vehicle	5/7	2204.16				
Ex82	8/8	1554.25	0.0082			60.8
Sunitinib	7/7	1255.6	< 0.0001	0.092		42.8
Ex82 + Sunitinib	7/7	879.12	< 0.0001	< 0.0001	0.008	20.1

Figure 4. Combination S1P1 and VEGF pathway inhibition reduces RCC tumor growth.

Tumor growth curves from the 786-O RCC tumor xenograft model are shown for the 4 treatment arms: vehicle, S1P1 antagonist (Ex82), sunitinib or the combination. The table shows that tumors from mice treated with sunitinib and the Ex82 grow more slowly than the vehicle treated tumors and that the addition of Ex82 to sunitinib adds to the tumor growth control of sunitinib (P=0.008).

Figure 5. Combination S1P1 and VEGF pathway inhibition lowers tumor blood flow.

Arterial Spin Labeled Magnetic Resonance Imaging (ASL MRI) blood flow images are shown for tumors from mice treated serially with vehicle (A) or Ex82 (B) at day 0, day 7 and day 21. For each image pair, the black and white image is the MRI anatomic image and the corresponding colored image is the ASL image. The tumor is circled with a yellow line and the area in yellow is the region of interest for which the blood flow is measured. The color scale corresponds to tumor blood flow values. Below the color scale are 2 representative images of tumors from mice treated with sunitinib or sunitinib + Ex82 and imaged on day 26 (+/- 4 days depending on availability of MR scanner) (C). Statistical analysis is shown in the accompanying graphs (D, E) and Table (E), which shows the P values for differences in tumor blood flow at day 26.

Figure 6. The combination of VEGFR and S1P1 inhibition induces tumor cell apoptosis. Multiplexed panels to assay tumor angiogenesis are shown. The percent area of tumor vessels (labeled with CD34; green) increased with Ex82 (6Aiii and 6Av) and sunitinib (Su) significantly reduced the percent area of vessels (6Aii and 6v), but further reduction in tumor vessels was not detected with the combination of Ex82 and sunitinib (6Av). Pericyte staining was assessed by SMA (red), Hoechst staining is shown in blue, and S1P1 is shown in yellow. S1P1 expression tended to increase with Ex82 (6Aiii and 6vi) and was significantly less with sunitinib and the combination treatment. Examination of the effects of treatment on

tumor cells showed that sunitinib treatment tended to increase apoptosis (TUNEL stain shown in red) and the combination treatment significantly increased apoptosis more than the vehicle or either of the single agents (6Biv and 6vi). CD34 staining is shown in green and Ki67 is shown in yellow. Hoechst staining is shown in blue. Whole tumor cross-sections are shown in 6C stained for TUNEL (gray). Bars represent mean $\pm$ SEM. * = $p < 0.05$ vs. vehicle, † = $p < 0.05$ vs. Ex82, ‡ = $p < 0.05$ vs. Su.

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Figure 7. Conceptual Role of S1P/S1P1 Signaling in Tumor Angiogenesis.

This model depicts our hypothesis about the effects of S1P1 and VEGFR inhibition. Panel A shows an abundant tumor vascular bed. VEGF pathway inhibition leads to decreased sprouting and a defect in the development of the vascular bed (B). S1P induces vascular sprouting. Thus, S1P1 inhibition leads to hypersprouting resulting in non-functional angiogenesis (C). Combination therapy leads to loss of S1P dependent vessels likely induced by VEGFR inhibition and the VEGF dependent hypersprouting induced by the S1P1 inhibitor (D).

Table 1.
Ex82 potently and selectively inhibits S1P1 beta arrestin activity.

Ex82 is a potent antagonist of both human and mouse S1P1 in a beta arrestin recruitment and GTPγS assay.

Assay	S1P1 Antagonist IC50 (nM)
Beta Arrestin Human S1P1	5.18+/-2.3
Beta Arrestin Mouse S1P1	4.0
Beta Arrestin Human S1P2	> 20,000
Beta Arrestin Human S1P3	>20,000
Beta Arrestin Human S1P4	>20,000
Beta Arrestin Human S1P5	>20,000

S1P1 antagonist Ex82 potently and selectively inhibits S1P1 beta arrestin activity.