Modulating Sphingosine-1-Phosphate receptors to improve chemotherapy delivery to Ewing sarcoma

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Abbreviations:

S1P= Sphingosine-1-Phosphate
S1PR= Sphingosine-1-Phosphate receptor
ES= Ewing Sarcoma
VEGF-A= Vascular Endothelial Growth Factor-A
Akt= Protein kinase B
ERK= Extracellular signal-regulated kinase
Rac1= RAS-related C3 botulinum toxin substrate 1
VE-cadherin= Vascular Endothelial-cadherin
ROCK1= Rho associated protein kinase1
PTEN= Phosphatase and tensin homologue
 α -SMA= alpha-smooth muscle actin
NG2= Neural/glial antigen2 Chondroitin Sulfate Proteoglycan
CAIX= Carbonic anhydrase IX
GLUT-1= Glucose Transport-1
HIF1 α = Hypoxia inducible factor 1 subunit alpha
GADPH= Glyceraldehyde3-phosphate dehydrogenase
PyMT =Polyoma middle T antigen
DOXO= Doxorubicin
SEW= SEW2871
JTE= JTE-013
DMSO=dimethyl sulfoxide
DAPI=4',6'-diamidino-2-phenylindole, dyhydrochloride
NF- κ B=Nuclear factor kappa light chain enhancer of activated B cells
FITC=Fluorescein isothiocyanate

NOVELTY AND IMPACT

This study demonstrates that Sphingosine-1-Phosphate (S1P) receptors are potential novel targets for tumor vasculature remodeling and adjuvant therapy for the treatment of Ewing Sarcoma. Unlike receptor tyrosine kinases that have already been extensively evaluated for use as vascular normalizing agents in oncology, S1P receptors are G protein-coupled receptors, which have not been well studied in tumor endothelium. Pharmacologic activators and inhibitors of S1P receptors are currently in clinical trials for treatment of auto-immune and cardiovascular diseases, indicating potential for clinical translation of this work.

ABSTRACT

Tumor vasculature is innately dysfunctional. Poorly functional tumor vessels inefficiently deliver chemotherapy to tumor cells; vessel hyper-permeability promotes chemotherapy delivery primarily to a tumor's periphery. Here we identify a method for enhancing chemotherapy delivery and efficacy in Ewing sarcoma (ES) in mice by modulating tumor vessel permeability. Vessel permeability is partially controlled by the G protein-coupled Sphingosine-1-phosphate receptors 1 and 2 (S1PR1 and S1PR2) on endothelial cells. S1PR1 promotes endothelial cell junction integrity while S1PR2 destabilizes it. We hypothesize that an imbalance of S1PR1:S1PR2 is partially responsible for the dysfunctional vascular phenotype characteristic of ES and that by altering the balance in favor of S1PR1, ES vessel hyper-permeability can be reversed. In this study, we demonstrate that pharmacologic activation of S1PR1 by SEW2871 or inhibition of S1PR2 by JTE-013 caused more organized, mature, and functional tumor vessels. Importantly, S1PR1 activation or S1PR2 inhibition improved chemotherapy delivery to the tumor and anti-tumor efficacy. Our data suggests that pharmacologic targeting of S1PR1 and S1PR2 may be a useful adjuvant to standard chemotherapy for ES patients.

Keywords: Ewing sarcoma, S1PR1, S1PR2, Vascular permeability, Chemotherapy efficacy

70 INTRODUCTION

71 Ewing sarcoma (ES) is an aggressive sarcoma of bone and soft tissue that represents 3% of all
 72 pediatric malignancies¹. ES tumor vessels, like vessels in many solid tumors, are characterized by a
 73 discontinuous endothelial lining with wide endothelial cell junctions and disjointed pericyte coverage.
 74 This contributes to marked vessel leakiness². Excessive vascular leakage causes chemotherapy
 75 extravasation at the periphery of the tumor, contributing to suboptimal chemotherapy efficacy².

76 Vascular remodeling, or normalization, using anti-angiogenic agents enhances perfusion and
 77 drug delivery in solid tumors³. A “normalized” vascular phenotype includes improved vessel function
 78 and reduced hyper-permeability; specifically, increased perivascular cell coverage and restored
 79 endothelial barrier integrity³. It was recently demonstrated that combination of anti-angiogenic agent
 80 celecoxib with standard chemotherapy improved survival in patients with metastatic ES⁴. Although this
 81 study indicates the potential of vascular remodeling as adjuvant therapy for ES, severe toxicities limited
 82 clinical usefulness of the anti-angiogenic agent.

83 Here, we present an alternative approach to improve drug delivery by targeting tumor vascular
 84 hyper-permeability. Several tumor secreted factors such as vascular endothelial growth factor-A
 85 (VEGF-A) cause disruption of adherens junctions by phosphorylation, internalization and cleavage of
 86 Vascular-Endothelial cadherin (VE-cadherin), an essential regulator of endothelial cell-cell adhesive
 87 properties⁵. Sphingosine-1-Phosphate (S1P) is a sphingolipid that regulates endothelial barrier function
 88 and angiogenesis via G protein-coupled S1P Receptors 1 and 2 (S1PR1 and S1PR2)⁶. S1PR1-G_i-Rac
 89 signaling decreases vessel permeability and enhances endothelial cell-to-cell junctions by inducing VE-
 90 cadherin trafficking to adhesion sites⁶. S1PR1 also regulates the interaction of perivascular and
 91 endothelial cells in microvasculature, creating more mature vessels⁷. Conversely, S1PR2 activation
 92 disrupts endothelial cell junctions via the G_{12/13}- Rho-Rho kinase kinase (ROCK)-PTEN pathway by
 93 preventing VE-cadherin translocation to cell contact sites⁸. The balance of S1PR1 and S1PR2 in a
 94 vascular bed thus defines endothelial barrier integrity⁸. In physiologic conditions, a high S1PR1: low
 95 S1PR2 ratio maintains a stabilized and intact vascular endothelium while a low S1PR1: high S1PR2
 96 ratio causes endothelial barrier dysfunction⁹. Although the antagonistic relationship between S1PR1

and S1PR2 is known in healthy endothelium and several disorders related to pathologic vascular permeability⁸, the role of these receptors in modulating tumor vasculature function is poorly understood.

We previously demonstrated that S1PR1 and S1PR2 are expressed on ES endothelium and that reduced tumor vessel hyper-permeability after exercise-induced shear stress correlates with increased S1PR1 and decreased S1PR2¹⁰. These findings suggest that ES vessel function might be improved by altering the balance in favor of S1PR1. To elucidate the role of S1PR1 and S1PR2 on tumor vasculature function, we performed preclinical studies in human ES mouse xenograft models using selective pharmacological modulators of S1PR1 and S1PR2. SEW2871 is a S1PR1-selective agonist that induces receptor internalization and subsequent recycling via AKT/ERK1/2/Rac1 pathway activation⁶. JTE-013 is an S1PR2 antagonist which prevent ROCK-PTEN pathway activation⁸.

Here, we demonstrate that activation of S1PR1 signaling or inhibition of S1PR2 signaling induced more normalized tumor vessels with reduced hyper-permeability. Importantly, vascular normalization by S1P receptor modulation correlated with significantly improved chemotherapy efficacy. This is the first study to demonstrate the role of S1P receptors in vascular function in ES. Our findings reveal a novel mechanism of tumor vascular remodeling via activation of S1PR1 and inhibition of S1PR2 signaling that contributes to increased chemotherapy delivery and, therefore, increased anti-tumor effects in mice.

MATERIAL AND METHODS

Cell culture

A673 ES cells (ATCC Cat# CRL-1598, RRID:CVCL_0080) were cultured per manufacturer recommendation. Cells are authenticated by STR analysis at MD Anderson Cancer Center Characterized Cell Line Core Facility within the last three years and routinely tested negative for mycoplasma contamination.

124 **Animals and experimental protocol**

125 The Institutional Animal Care and Use Committee at The University of Texas MD Anderson
126 Cancer Center approved the animal studies. A673 cells (2.5×10^6) were injected subcutaneously into the
127 backs of 6-week-old athymic nude (nu/nu) male mice. After tumors reached $\sim 50 \text{ mm}^3$, mice were
128 randomized into 4 groups: daily oral vehicle (dimethyl sulfoxide [DMSO] or alcohol), daily oral
129 pharmaceutical agent (SEW2871 10mg/kg or JTE-013 1.5mg/kg; Cayman Chemical), intravenous
130 doxorubicin (2mg/kg twice per week; Premier Pharmacy), and combination therapy with pharmaceutical
131 agent + doxorubicin. The SEW2871 experiment was repeated in an orthotopic model using the same
132 treatment schedule in which A673 cells (2.5×10^5) were injected into the gastrocnemius of 6-week-old
133 nude mice. Mice were housed in individually ventilated cages under pathogen-free conditions. Animals
134 had free access to food and water and were kept on a 12-hour light/12-hour dark cycle.

135

136 **Vessel structure and function**

137 Five minutes prior to euthanasia, tumor-bearing mice were injected via tail vein with 100 μ L
138 tomato-lectin (2mg/mL in PBS 7.4, VectorLab) or high molecular weight FITC-Dextran (2,000,000 mol
139 weight, 10mg/mL in PBS pH 7.4; Sigma-Aldrich).

140 Frozen tumor sections were stained with the following primary antibodies: rat anti-CD31 (1:50,
141 BD Pharmingen), rabbit anti- α -smooth muscle actin (α -SMA, 1:100, Abcam Ab5694;), rabbit anti-
142 NG2 Chondroitin Sulfate Proteoglycan (NG2, 1:100, AB5320 Millipore), mouse rat anti-Vascular
143 Endothelial cadherin (Ve-caderin, 1:100, BD Pharmingen). Nuclei were stained with Fluoro-Gel II with
144 DAPI (Electron Microscopy Sciences). Images were captured with a Leica DM5500 B upright
145 microscope imaging system (Leica Microsystems) and analyzed using NIS-Elements Imaging Software.
146 For all immunostaining assays, 5 random fields from each tumor sample were quantified as previously
147 described¹¹. Images for VE-cadherin and CD31 staining were obtained with a 63x oil immersion
148 objective using a Zeiss LSM 880 with Airyscan FAST confocal microscope. Pearson's correlation
149 coefficient was calculated to measure the colocalization correlation of the intensity distribution between
150 VE-cadherin and CD31 of 5 random vessels per tumor sections.

151 Hypoxia

152 qPCR was performed with iQ SYBR[®] Green Supermix (Bio-Rad) and run on a LightCycler[®] 480
 153 Instrument II (Roche). Carbonic anhydrase IX (CAIX) (FW: GAGAAGGCAGCACAGAAG G and REV:
 154 GGCTTCTCACATTCTCCAAGAT) and Glucose Transport-1 primers (GLUT-1) (FW: GGGCCA
 155 AGAGTGTGCTAAA and REV: CTTCTTCTCCCGCATCATCTG), Vascular Endothelial Growth Factor-A
 156 (VEGF-A) (FW: GTGAATGCAGACCAAAGAAAGATA G and REV: CCAGGACTTATACCGGGATTTC);
 157 Hypoxia inducible factor 1 subunit alpha-1 α (HIF-1 α) (FW: GTCTGCAACATGGAAGGTATTG and
 158 REV: GCAGGTCATAGGTGGTTTCT) as well as internal control primers Glyceraldehyde-3-phosphate
 159 dehydrogenase (GAPDH) (FW: AACAGCAACTCCCACTCTTC and REV:
 160 CCTGTTGCTGTAGCCGTATT) were synthesized by Integrated DNA Technologies.

161

162 Proliferation assay (Live-cell imaging)

163 A673 cells (5×10^3 per well) were grown in a 96-well plate and filmed every four hours for 48
 164 hours with Incucyte live cell imaging system (Essen Instrument., Ann Harbor, MI). Doxorubicin
 165 (0.01nM), SEW2871 (50nM), or JTE-013 (50 μ M) was added at the beginning of the quantification
 166 period, or DMSO or ethanol as a negative control. The experiment was performed three times.
 167 Proliferation was monitored by analyzing the cell occupied area (% confluence) of images over time.

168

169 Statistical analysis

170 All values are reported as means $\pm$ standard error of the mean. Statistical significance of the results
 171 was calculated by two-way analysis of variance. Intergroup differences were evaluated by using
 172 student's t-test and linear mixed models. The statistical analysis was performed using SpSS (version
 173 21) and Graphpad software.

174

175 All data and detailed methods will be made available upon reasonable request.

176

RESULTS

S1PR1 activation by SEW2871 promotes tumor vascular normalization.

To determine the effect of S1PR1 activation in ES, A673 subcutaneous and orthotopic xenografts were established in nude mice. Mice were treated with an S1PR1 selective agonist, SEW2871, alone or in combination with doxorubicin. There were no significant differences in microvessel density or total vessel count between tumors in different treatment groups in either subcutaneous or orthotopic tumors (data not shown). In both models, S1PR1 activation promoted tumor vessel normalization. SEW2871 treatment stimulated the formation of more elongated vessels with a greater number of open lumens in subcutaneous tumors (Elongated Vessels: SEW $p=0.016$, Lumens: SEW $p=0.046$, Fig. 1A and C) and orthotopic tumors (Elongated Vessels: SEW $p=0.014$, Lumens: SEW $p=0.013$, Fig. 1B and D). Further, S1PR1 activation significantly increased mural cell coverage of tumor vessels. The percentage of tumor capillaries with observable alpha-smooth-muscle actin (α -SMA) (SEW $p=0.002$, Fig. 1E) and Neuron-glia 2 (NG2) labeling (SEW $p=0.046$, Fig. 1F) was significantly increased in SEW-treated subcutaneous tumor sections compared to untreated tumors. Similarly, but with a less significant effect, α -SMA (SEW $p=0.061$, Fig. 1E) and NG2 (Fig. 1F, SEW $p=0.065$) coverage of capillaries was also increased in SEW-treated orthotopic tumors.

Tumor vascular permeability was assessed by injection of high molecular weight FITC-dextran (2000 KDa), a molecule that does not leak from functional vessels¹². S1PR1 activation caused a 34% reduction in vascular leakiness of subcutaneous tumor vessels that became more evident in doxorubicin-treated mice (87%; DOXOxSEW $p=0.005$, Fig. 1G). In the orthotopic model, a significant reduction in dextran leakage was observed in both tumors treated with SEW2871 alone (53%) or in combination with doxorubicin (52%; SEW $p=0.004$, Fig. 1G).

To confirm improvement in barrier integrity by the S1PR1 activation, we analyzed VE-cadherin protein expression and localization at the plasma membrane by confocal microscopy analysis. Although SEW2871 treatment did not change the total VE-cadherin expression levels (data not shown), it did significantly increase the translocation of VE-cadherin at the adhesion sites, demonstrated by co-localization with CD31 in both subcutaneous and orthotopic tumors (Fig. 1H-I).

S1PR2 inhibition by JTE-013 promotes tumor vasculature normalization.

To study the impact of S1PR2 inhibition on tumor angiogenesis, mice bearing subcutaneous A673 tumors were treated with JTE-013, doxorubicin, or the combination of JTE-013 and doxorubicin. Inhibition of S1PR2 caused a significantly higher microvessel density ($p=0.027$) that appeared more elongated ($p=0.068$) with a greater number of open lumens ($p=0.067$) in subcutaneous tumors (Fig. 2A, C). There was no significant effect of JTE-013 in remodeling microvessel structure and organization in orthotopic tumors (data not shown). Although α -SMA (Fig. 2A, D) and NG2 cell coverage did not change (Fig. 2A, E), JTE-013 reduced tumor vessel leakage by 68% and 43.7% in subcutaneous and orthotopic tumors, respectively ($p=0.038$, Fig. 2F and 2G). Additionally, while the pharmacological inhibition of the S1PR2 pathway did not affect VE-cadherin translocation at the plasma membrane (data not shown), we observed a trend toward S1PR2 antagonism increasing VE-cadherin protein expression levels in both subcutaneous (SEW $p=0.064$, Fig. 2H-I) and orthotopic tumors (SEW $p=0.071$, Fig. 2H-I).

S1PR1 activation or S1PR2 inhibition improved chemotherapy efficacy.

The vascular remodeling observed after SEW2871 treatment correlated with a 2-fold increase (51%) in the number of functional (lectin perfused) vessels compared to control tumors ($p=0.028$, Fig. 3A). Consistent with improved vascular function leading to reduced tumor hypoxia, SEW2871 significantly reduced CaIX (46%; SEW $p=0.043$, Fig. 3B) and VEGF-A mRNA levels (71%; SEW $p=0.045$) and had a clear trend toward reduced GLUT-1 (63%, Fig. 3B) and HIF1- α mRNA in orthotopic tumors (33%, Fig. 3B). Improved tumor perfusion and reduced hypoxia should improve chemotherapy delivery. Indeed, SEW2871 plus chemotherapy inhibited A673 tumor growth better than chemotherapy alone (subcutaneous: 41% better, Time: DOX+SEW vs DOX $p<0.0001$; and orthotopic: 45% better, Time: DOX+SEW vs DOX $p<0.0001$, Fig. 3C). No additive effect of SEW2871 and doxorubicin in inhibiting proliferation of A673 cells was observed *in vitro* (Fig. 3D).

Unlike S1PR1 activation, S1PR2 pathway inhibition did not significantly change CaIX, GLUT-1, HIF1- α , VEGF-A mRNA levels in orthotopic tumors (Fig. 3E). Still, tumors treated with the combination

of JTE-013 and doxorubicin were significantly smaller than tumors treated with doxorubicin alone in both xenograft models (subcutaneous: 43.9%, DOX+JTE vs JTE $p=0.02$; orthotopic 49.8% better, $p=0.03$) (Fig. 3F). JTE-013 treatment did not enhance the cytotoxicity of doxorubicin in A673 cells *in vitro* (Fig. 3G).

DISCUSSION

Here, we provide evidence that modulating the ratio of S1PR1:S1PR2, by inhibiting S1PR1 or activating S1PR2, may be a novel method to remodel tumor vasculature and increase the delivery and thus the efficacy of chemotherapy.

In ES tumors, the activation of S1PR1 preferentially manifested as more elongated and open lumen vessels markedly covered with mural cells. Instead of promoting vascular sprouting and neoformation as previously reported in PyMT breast cancer¹³, the activation of S1PR1 by the agonist SEW2871 principally remodeled the morphology of ES tumor vessels to a more mature phenotype. This different vascular response may be partially dependent on tumor type. In comparison to other tumor types, ES vascular network is unique as it is characterized by a more morphologically organized vascular network¹⁰. However, consistent with other tumors, vasculature is dysfunctional and hyper-permeable.

The inhibition of S1PR2 by the antagonist JTE-013 only marginally increased the microvessel density, consistent with a previous report in S1PR2^{-/-} mice bearing Lewis Lung carcinoma and B16BL6¹⁴. Since more significant effects on vascular remodeling was observed by activating S1PR1, the inhibition of S1PR2 by pharmacological modulation might be not sufficient enough to overcome the vascular dysfunction largely caused by lack of S1PR1 pathway activations in ES tumors.

In addition, we demonstrated that improved endothelial cells junction function might be involved in tumor vascular normalization. Activation of S1PR1 reduced leakage of tumor vessels by stimulating the recruitment of mural cells around the endothelium and increasing VE-cadherin localization at endothelial junctions. Inhibition of S1PR2 also decreased vascular hyper-permeability, but the mechanism by which this occurs is less clear, as changes to VE-cadherin localization were subtle. The

trend toward increased VE-cadherin expression after inhibition of S1PR2 is consistent with previous reports that S1PR2 signaling via NF- κ B¹⁵ promotes the expression of the zinc-finger transcription factor Snail that represses VE-cadherin transcription in endothelial cells exposed to cancer cell-conditioned media^{16,17}.

In addition, we demonstrated that the S1PR1 agonism by SEW2871 contributes to increased blood vessel perfusion and oxygenation of the tumor. Lower levels of hypoxia responsive transcripts, including VEGF α , confirm a reduction in hypoxia¹⁸. Considering that lower levels of VEGF α result in more stable endothelial cell adhesion junctions^{5,19}, S1PR1 activation may alternatively increase vessel function as a result of decreased tumor hypoxia.

Moreover, since hypoxia induces cellular adaptations that promote cell survival and resistance to chemotherapy²⁰, the reduced hypoxic environment likely contributes to a greater anti-tumor response. By using athymic nude mice, which lack functional T-cells, we removed any possible effects of S1P receptor signaling modulation on lymphocytes trafficking and immune regulation²¹. Therefore, the increased chemotherapy efficacy observed by the combined therapy was likely endothelial cell dependent.

Our findings demonstrate that S1PR1 and S1PR2 in ES vasculature can be modulated to normalize tumor vessels and improve chemotherapy efficacy. As such, the pharmacologic targeting of S1PR1 and S1PR2 warrants further study as potential adjuvant therapy targets for Ewing sarcoma patients.

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FIGURES

Figure 1. S1PR1 activation by SEW2871 promotes tumor vascular normalization. After A673 tumor cells injection (7 days post-subcutaneous injection and 11 days post-intramuscular injection), tumor-bearing mice were treated with doxorubicin [DOX] (2mg/Kg, twice per week, intravenously) and/or SEW2871 [SEW] (S1PR1 agonist, 10mg/Kg, daily, orally). **(A and B)** α -SMA (red) or NG2 (red) and CD31 (green) or FITC-dextran leak (green) and CD31 (red) immunofluorescence with DAPI staining (nuclei) in subcutaneous **(A)** and orthotopic tumors **(B)**; scale bar: 100 μ m. **(C and D)** The number of visible lumens and the number of vessels>100 μ m (large) were counted in 5 random sections/tumor. **C. Subcutaneous.** Bars show means $\pm$ SEM, $n=5-6$. Two-way ANOVA, open lumens (SEW $p=0.0465$), vessels>100 μ m (SEW $p=0.016$, DOX $p=0.001$). **D. Orthotopic.** Bars show means $\pm$ SEM, $n=5-7$. Two-way ANOVA, open lumens (SEW $p=0.014$), vessels>100 μ m (SEW $p=0.015$, DOX $p<0.0001$). **(E)** Mean α SMA:CD31 ratio $\pm$ SEM calculated in 5 random sections/tumor. *Subcutaneous*, two-way ANOVA (SEW $p=0.002$), $n=5-6$. *Orthotopic*, two-way ANOVA (SEW $\times$ DOX $p=0.047$), $n=5-6$. Post-hoc Tukey test and indicated by # $p=0.061$. **(F)** Mean NG2:CD31 ratio $\pm$ SEM calculated in 5

375 random sections/tumor. *Subcutaneous*, two-way ANOVA (SEW $p=0.046$, DOX $p=0.068$), $n=4-5$.
 376 *Orthotopic*, two-way ANOVA (SEW $p=0.065$), $n=5-6$. **(G)** Mean Dextran:CD31 $\pm$ SEM ratio for individual
 377 A673 tumors. *Subcutaneous*, two-way ANOVA (SEW $p=0.009$, DOXxSEW $p=0.050$), $n=4-7$. Post-hoc
 378 Tukey test and indicated by $**p<0.01$, $***p<0.001$. *Orthotopic*, two-way ANOVA (SEW $p=0.004$), $n=5-8$.
 379 **(H)** Representative confocal images of subcutaneous and orthotopic tumors showing colocalization of
 380 VE-cadherin (red) and CD31 (green). Nuclei were counterstained with DAPI (blue). Scale bar $10\mu\text{m}$. Part
 381 of the section (white rectangle) is shown below in higher magnification (300x). **(I)** Graph shows
 382 Pearson's correlation coefficient for VE-cadherin and CD31 markers in subcutaneous and orthotopic
 383 tumors represented in Figure 1H. For each tumor 5 vessels were analyzed in different optical regions.
 384 *Subcutaneous*, two-way ANOVA (DOXO $p=0.044$, SEW $p=0.044$). *Orthotopic*, two-way ANOVA (SEW
 385 $p=0.0003$).

386

387 **Figure 2. S1PR2 receptor inhibition by JTE-013 promotes tumor vasculature normalization.** After
 388 A673 tumor cells injection (7 days post-subcutaneous injection and 13 days post-intramuscular
 389 injection), tumor-bearing mice were treated with doxorubicin [DOX] (2 mg/Kg, twice per week, i.v.)
 390 and/or JTE-013 [JTE] (S1PR2 antagonist, 2.5mg/Kg daily in subcutaneous model and 5mg/Kg twice a
 391 day in orthotopic model, orally). **(A, B)** α -SMA (red) or NG2 (red) and CD31 (green) or VE-cadherin in
 392 subcutaneous **(A)** and orthotopic tumors **(B)**; scale bar: $100\mu\text{m}$. **(C)** The average number of
 393 microvessels density (MVD), total vessels, the number of visible lumens and the number of
 394 vessels $>100\mu\text{m}$ (large) were counted in 5 random sections/subcutaneous tumor. Bars show means $\pm$
 395 SEM, $n=5-6$. Two-way ANOVA, MV (JTE $p=0.023$); total vessels ($p=\text{non-significant}$); open lumens (JTE
 396 $p=0.067$); vessels $>100\mu\text{m}$ (JTE $p=0.068$, DOX $p=0.103$). **(D)** Mean α SMA:CD31 ratio $\pm$ SEM calculated
 397 in 5 random sections/tumor. *Subcutaneous*, two-way ANOVA (DOXO $p=0.061$), $n=5$. *Orthotopic*, two-
 398 way ANOVA ($p=\text{ns}$), $n=5-6$. **(E)** Mean NG2:CD31 ratio $\pm$ SEM calculated in 5 random sections/tumor.
 399 *Subcutaneous*, two-way ANOVA (DOXO $p=0.109$), $n=4-6$. *Orthotopic*, two-way ANOVA (JTE $p=0.107$),
 400 $n=5-6$. **(F)** Representative images of FITC-dextran leak (green) and CD31 (red) immunofluorescence

with DAPI staining (nuclei) in orthotopic and subcutaneous tumors; scale bar: 100 μ m. **(G)** Mean Dextran:CD31 $\pm$ SEM ratio for individual A673 tumors. *Subcutaneous*, $n=5-7$, T-test Student $*p=0.038$. *Orthotopic*, T-test Student $*p=0.027$, $n=5-6$. **(H)** Representative images of subcutaneous and orthotopic tumors. VE-cadherin (red), DAPI staining (nuclei); scale bar: 100 μ m. **(I)** Quantification of VE-cadherin by mean fluorescence intensity (MFI) calculated in 5 random sections/tumor. Values represent means $\pm$ SEM. *Subcutaneous*, two-way ANOVA (JTE $p=0.071$), $n=5-6$. *Orthotopic*, two-way ANOVA (JTE $p=0.064$), $n=5-6$.

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Figure 3. S1PR1 activation by SEW2871 or S1PR2 inhibition by JTE-013 improves chemotherapy efficacy. **(A)** Mean Tomato-lectin:CD31 $\pm$ SEM ($n=4$) ratio defined the percentage of perfused vessels, t-test $*p=0.028$. **(B)** RT-PCR analysis of the mRNA expression levels of CaIX, GLUT-1, HIF1- α , VEGF-A normalized against GAPDH in orthotopic tumor homogenates. Data are expressed as the mean $\pm$ SEM of triplicate values. Bars represent means $\pm$ SEM, $n=5-6$. Statistical significance of the results was calculated by two-way analysis of variance (CaIX: SEW $p=0.043$; GLUT-1: SEW $p=0.0511$; VEGF-A: SEW $p=0.045$; HIF-1 α : SEW $p=0.078$). **(C)** A673 tumor volumes from mice treated with [DOX] and/or [SEW] were measured in indicated days. *Subcutaneous*. Values are means $\pm$ SEM for 6-7 animals in each group. Linear Mixed Model (Time: SEW vs CON $***p<0.0001$; Time: DOX+SEW vs CON $*p=0.0131$ and Time: DOX+SEW vs DOX $***p<0.0001$). *A673 Orthotopic*. Values are means $\pm$ SEM for 5-8 animals in each group. Linear Mixed Model (TIME: CON vs DOX $*p<0.05$; Time: CON vs SEW $**p<0.01$; Time: DOX+SEW vs CON $***p<0.0001$). **(D)** A673 cell proliferation assay (expressed as % of cell confluence) treated with SEW2871 (50nM) and doxorubicin (0.1nM) and combination of the two drugs. Linear Mixed Model: CON vs SEW $***p<0.001$; CON vs DOXO $***p<0.001$. **(E)** RT-PCR analysis of the mRNA expression levels of CaIX, GLUT-, HIF-1 α , VEGF-A normalized against GAPDH in orthotopic tumor homogenates. Data are expressed as the mean $\pm$ SEM of triplicate values. Bars represent means $\pm$ SEM, $n=5-6$. Statistical significance of the results was calculated by two-way ANOVA (CaIX: $p=ns$; GLUT-1: $p=ns$; VEGF-A $p=ns$; HIF-1 α $p=ns$). **(F)** A673 tumor volumes from mice

427 treated with [DOX] and/or [JTE] were measured in indicated days. *Subcutaneous*. Values are means $\pm$
 428 SEM for 6-8 animals in each group. Linear Mixed Model: Time: DOXO+JTE vs JTE *p=0.020.
 429 *Orthotopic*. Values are means $\pm$ SEM for 5-7 animals in each group. Linear Mixed Model: CON vs DOX
 430 p=0.016; DOX vs DOXO+JTE p=0.032. **(G)** A673 cell proliferation assay (expressed as % of cell
 431 confluence) when treated with JTE-031 (50 μ M) and DOX (0.1nM) and combination of the two drugs.
 432 Linear Mixed Model: CON vs DOX *p<0.05; JTE vs DOX+JTE **p<0.01.

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Figure 1. S1PR1 activation by SEW2871 promotes tumor vascular normalization .

Figure 2. S1PR2 inhibition by JTE-013 promotes tumor vasculature normalization .

Figure 3. S1PR1 activation by SEW2831 or S1PR2 inhibition by JTE-013 improved chemotherapy efficacy.