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Combined effect of vascular-leakage-blocker Sac-1004 and antiangiogenic drug sunitinib on tumor angiogenesis



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ABSTRACT

Tumor blood vessels are often leaky because of poor covering by mural cells and loose cell-to-cell contacts. Leaky vessels result in hemorrhage and limited vascular perfusion, which lead to hypoxic tumor microenvironment. Antiangiogenic agents have been shown to normalize the tumor blood vessels, albeit temporarily. Continued administration has been found to be associated with increased tumor hypoxia, a major driving force behind chemoresistance and metastasis. Sac-1004 was recently demonstrated to prevent vascular leakage, normalize tumor vessels and prevent metastasis in sustained manner. Here, we sought that combining antiangiogenic agent, sunitinib with Sac-1004 could have better inhibitory effect upon tumor growth. We found that B16F10 tumor growth was significantly reduced and tumor-bearing mice survival was increased upon combining sunitinib therapy with Sac-1004. In concordance with this observation, tumor vascular perfusion was substantially improved in tumors receiving combination therapy. In addition, tumor vascular leakage was reduced to higher extent in combination treatment group as compared to either therapy alone, an effect attributed to improved vascular junction. Interestingly, hypoxia in tumor environment was significantly reduced, when sunitinib was combined with Sac-1004. Taken together, our data demonstrates that combining antiangiogenic therapy with vascular-leakage inhibiting agent might be a beneficial strategy to combat cancer.

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1. Introduction

Angiogenesis, growth of new blood vessels from preexisting ones, is a highly coordinated essential event for physiologic as well as pathologic development [1]. The balance between pro- and anti-angiogenic factors is tightly regulated in physiological angiogenesis; however, this balance is tipped in pathologic conditions as tumor [2,3]. Tumor angiogenesis is characterized by irregular, dilated, saccular, tortuous and leaky vessels [4–6], which leads to

Abbreviations: DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; OCT, optimal cutting temperature; PFA, paraformaldehyde; DAPI, 4',6-diamidino-2-phenylindole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor; PDGFR, platelet derived growth factor receptor; FLT, fms-like tyrosine kinase; RET, rearranged during transfection; CSF, colony-stimulating factor.

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hypoxic and acidotic microenvironment as a result of abnormal blood flow [7]. Such an adverse environment enhances the chemoresistance of tumor cells and enables them to migrate to distant sites causing metastasis [5,6].

Antiangiogenic therapy is widely being used to treat a wide variety of tumors. Amelioration of amateur blood vessels in tumor by antiangiogenic agents leaves more mature ones behind, which provides a normalization effect [3,4]. This state provides a window of opportunity to co-administered cytotoxic therapy resulting in enhanced anti-tumor activity [8,9]. Even though this approach is quite promising one, the major drawback is that continued administration of antiangiogenic agents often wipes out tumor vessels below a critical level, causing hypoxia to build-up. This inadvertent event has been shown to enhance regional and distal metastasis in tumor-bearing subjects [10,11]. The antiangiogenic therapy could be much beneficial if hypoxia buildup in tumor environment could be accounted for.

Recently, we demonstrated that a vascular-leakage blocker, Sac-1004 could reliably be used to reduce tumor vascular

hyperpermeability, induce vessel normalization and prevent metastasis [12]. Sac-1004 was able to wane vascular hyperpermeability by reorganizing the actin cytoskeleton, specifically in endothelial cells, to form cortical actin ring via cAMP/Rac/Cortactin pathway [13,14]. This stabilization of actin ring was related to localization of adhesion-junction protein VE-cadherin at cell–cell junction. We showed that Sac-1004 could sustainably reduce tumor hypoxia and modulate tumor environment in order to dampen epithelial-to-mesenchymal transition and tumor aggressiveness [12].

Here, we have combined antiangiogenic treatment with Sac-1004 envisioning better therapeutic outcome. Administration of Sac-1004 to sunitinib-receiving B16F10 tumor-bearing-mice significantly reduced tumor growth as compared to either therapy alone. Moreover, greater inhibition of hypoxia was observed in tumors receiving combination therapy. Thus, combination therapy of tumor with antiangiogenic agent and vascular-leakage-inhibitor could be an attractive approach to substantially improve therapeutic efficiency.

2. Materials and methods

2.1. Drugs

Sac-1004 was synthesized as described previously [14]. Stock solution of Sac-1004 (50 mg/ml) was prepared in dimethyl sulfoxide (DMSO), and dilutions were made in phosphate buffered saline (PBS). Sunitinib malate was purchased from Cayman Chemicals CoA, Korea. Stock solution of sunitinib (5 mg/ml) was in DMSO and PBS was used for dilutions.

2.2. Mice

C57BL/6J mice (7 weeks old) were bought from Daehan Biolink (Seoul, Korea) and maintained in a laminar airflow cabinet under specific pathogen-free conditions. The facilities were approved by the Association of Assessment and Accreditation of Laboratory Animal Care, and animal experiments were conducted under the institutional guidelines established for the Animal Core Facility at Yonsei University College of Medicine with approval of the institutional care and use committee.

2.3. Tumor model and treatment regime

Tumors were subcutaneously established by injecting B16F10 cells (5×10^5 cells; 100 μ l) on the lateral flank of 8-week-old C57BL/6J mice. Sac-1004 (50 mg/kg) or an equivalent volume of DMSO (in PBS; 100 μ l) was administered intravenously daily for 7 or 14 days, as indicated in the study plan. Mice receiving combination therapy or sunitinib alone were intraperitoneally injected with sunitinib (20 mg/kg) every third day for 2 weeks. Tumor volumes measurement was done daily with calipers and calculated as $\text{width}^2 \times \text{length} \times 0.523$. Tumors were captured at a volume of 1000 mm³ for almost all of the experiments except growth curve and survival curve experiment, where tumor growth was monitored for 2 weeks or more.

2.4. Tumor permeability and vascular perfusion

Evans blue and fluorescein isothiocyanate (FITC)-dextran was used to identify tumor vascular leakage as previously described [15]. Evans blue dye (50 mg/kg) was injected intravenously 30 min prior to tumor excision. The tumor tissues were dried at 60 °C for 16 h and the dye was extracted with 1 ml of formamide at 55 °C for 16 h. Absorbance was taken at 620 nm. For FITC-dextran-mediated leakage assessment, an intravenous injection of

3 mg/mouse FITC-dextran (40-kDa; Sigma Aldrich) was made 10 min before capture of tumor. Tumors were then fixed briefly in 4% paraformaldehyde (PFA) and embedded in optimal cutting temperature (OCT) compound. Sections of 50 μ m thickness were then directly viewed under fluorescence microscope to identify vascular leakage.

Vessel perfusion was quantified using biotinylated *Lycopersicon esculentum* (tomato) lectin [16], (0.1 mg/mouse; Vector Laboratories) injected intravenously 10 min before excision of tumor.

2.5. Histology and immunostaining

Tumor-bearing mice were anaesthetized and were perfused with 1% PFA. Tumors were captured and after a brief incubation in 4% PFA incubated in 15% and 30% sucrose solutions. Specimens were embedded in OCT compound and sectioned at 20 μ m thickness. Staining was performed as previously described [8]. Primary antibodies used were: rat anti-CD31 (1:100; BD Pharmingen, Korea), goat anti-VE-cadherin (1:100; Santa Cruz) or rabbit anti-NG2 (1:500; Millipore, Korea). The sections were then incubated in Alexa Fluor-conjugated secondary antibodies. 4',6-diamidino-2-phenylindole (DAPI, 1 ng/ml) was used to stain nuclei. Sections were then photographed with a confocal microscope (Zeiss LSM 510) or an Olympus IX81-ZDC inverted fluorescence microscope.

Hypoxia in tumor environment was detected using pimonidazole (75 mg/kg; Hypoxyprobe-1, Chemicon) injected intravenously 1 h before capture of tumor.

2.6. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Cells were seeded at a density of 2×10^4 cells (HUVEC; human umbilical vein endothelial cell) or 1×10^4 cells (B16F10) on 24-well plates. After overnight incubation, cells were starved with 1% M199 media for 4 h and then treated with various concentrations of Sac-1004 and sunitinib. After 24 h, cells were washed and 0% M199 media containing MTT (0.1 mg/ml) was added followed by incubation at 37 °C for 3 h. The formazan crystals were dissolved in 200 μ l of DMSO:Ethanol (1:1). The absorbance was measured at 560 nm.

2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software, La Jolla, CA). Tests for statistical significance were two-sided, and probability values less than 0.05 were considered significant. The Student's *t*-test or ANOVA was used to compare mean values, and results are presented as mean \pm SEM or SD.

3. Results

3.1. Combination treatment of Sac-1004 and sunitinib reduces tumor growth and increases survival

Previously, we showed that Sac-1004 was able to enhance the accessibility of cytotoxic drug cisplatin to tumor cells and result in decreased tumor growth upon combination therapy [12]. Also, sunitinib has been shown to antagonize tumor growth by wiping out tumor blood vessels [17]. So, we sought that combining Sac-1004 and sunitinib might have better therapeutic outcome. Use of sub-optimal dose of sunitinib decreased the growth rate of B16F10 tumor and combining sunitinib with Sac-1004 further lowered the growth rate significantly (Fig. 1B). Analysis of end-point tumor volume showed that tumor volume was substantially reduced in combination treatment group as compared to sunitinib treatment alone (Fig. 1C).

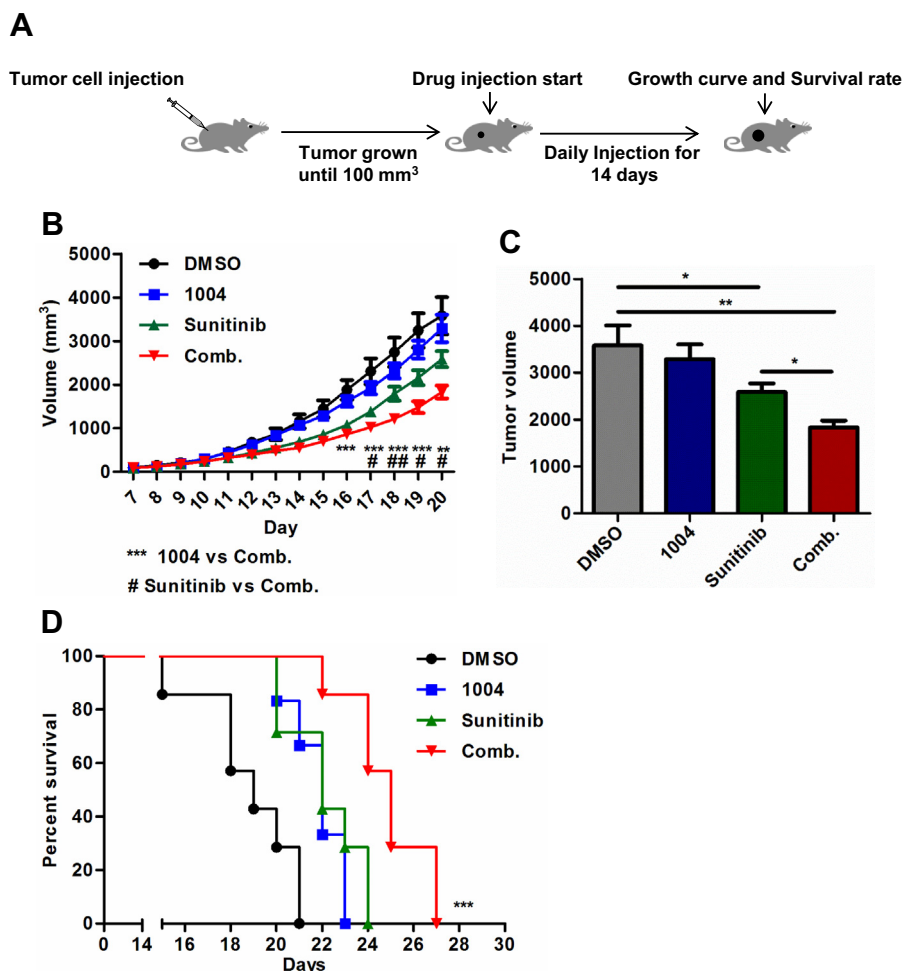


Fig. 1. Combination treatment of Sac-1004 and sunitinib reduces tumor growth and increases mice survival. (A) Schematics for the treatment of B16F10 tumor with Sac-1004, sunitinib or combination. (B) Growth curve of B16F10 tumors subjected to therapies as indicated. Tumor volume was measured everyday over the experimental time course ($n = 8$ mice per group). (C) Comparison of final tumor volumes of each group. Data represent mean \pm SEM. * $P < 0.05$; ** $P < 0.01$. (D) Survival curve of tumor-bearing mice receiving various therapies as indicated. Each point in Kaplan–Meier curve represents percent of surviving mice. Mice with tumor size greater than 5000 mm³ were considered dead. The p values were calculated using log rank test ($n = 8$ mice per group). *** $P < 0.001$.

We also analyzed the survival of B16F10 tumor-bearing mice that received Sac-1004 and sunitinib therapy. As it was reported previously [18], mice with tumor size greater than 5000 mm³ were considered dead and sacrificed. Mantel-cox test of survival curve data clearly showed that tumor-bearing mice receiving combination therapy of Sac-1004 and sunitinib had significantly increased life-span (Fig. 1D). Thus, our data demonstrates that combination treatment of Sac-1004 and sunitinib could increase the survival of tumor-bearing mice.

3.2. Sac-1004 increases the effect of sunitinib on blood vessels functions

Reduction in tumor growth suggests better delivery of sunitinib to tumor interiors. Indeed, Sac-1004 was shown to enhance the vascular patency in different tumor types [12]. So, we analyzed the effect of combination therapy on B16F10 tumor blood vessel functionality. Lectin was injected intravenously to mice prior to capture of tumor to identify the ratio of perfused blood vessels. Sac-1004 treated tumors, as demonstrated before [12], had increased number of perfused vessels as compared with control tumor (Fig. 2B and C). Sunitinib alone treatment however, did not show much increase in vascular patency. Upon combination

with Sac-1004, we saw significant increase in the number of perfused blood vessels (Fig. 2B and C). Though no visible difference in perfusion was observed between Sac-1004 and combination group, we would like to emphasize that Sac-1004 did increase the effectiveness of sunitinib in improving vessel functionality.

3.3. Combination treatment of Sac-1004 and sunitinib reduces tumor vessels leakage synergistically

Several factors including reduction in vascular leakage could result in improvement of vascular functionality. Since Sac-1004 had exhibited the potential to decrease tumor vascular leakage [12], and sunitinib has also been reported to antagonize vascular hyperpermeability [8], we analyzed the effect of combination treatment on B16F10 tumor-vessel leakiness. Both Sac-1004 and sunitinib were able to reduce leakage in tumor blood vessels, as demonstrated by Evans Blue method, to similar extent (Fig. 2D). However, upon combination treatment, leakage reduction observed was significantly greater than either treatment alone (Fig. 2D). This quantitative analysis was visually reconfirmed using FITC-dextran. Fig. 2E clearly shows exudation of FITC-dextran from leaky vessels in DMSO group. However, leakage was much reduced in Sac-1004/sunitinib/combination groups (Fig. 2E and F). Thus

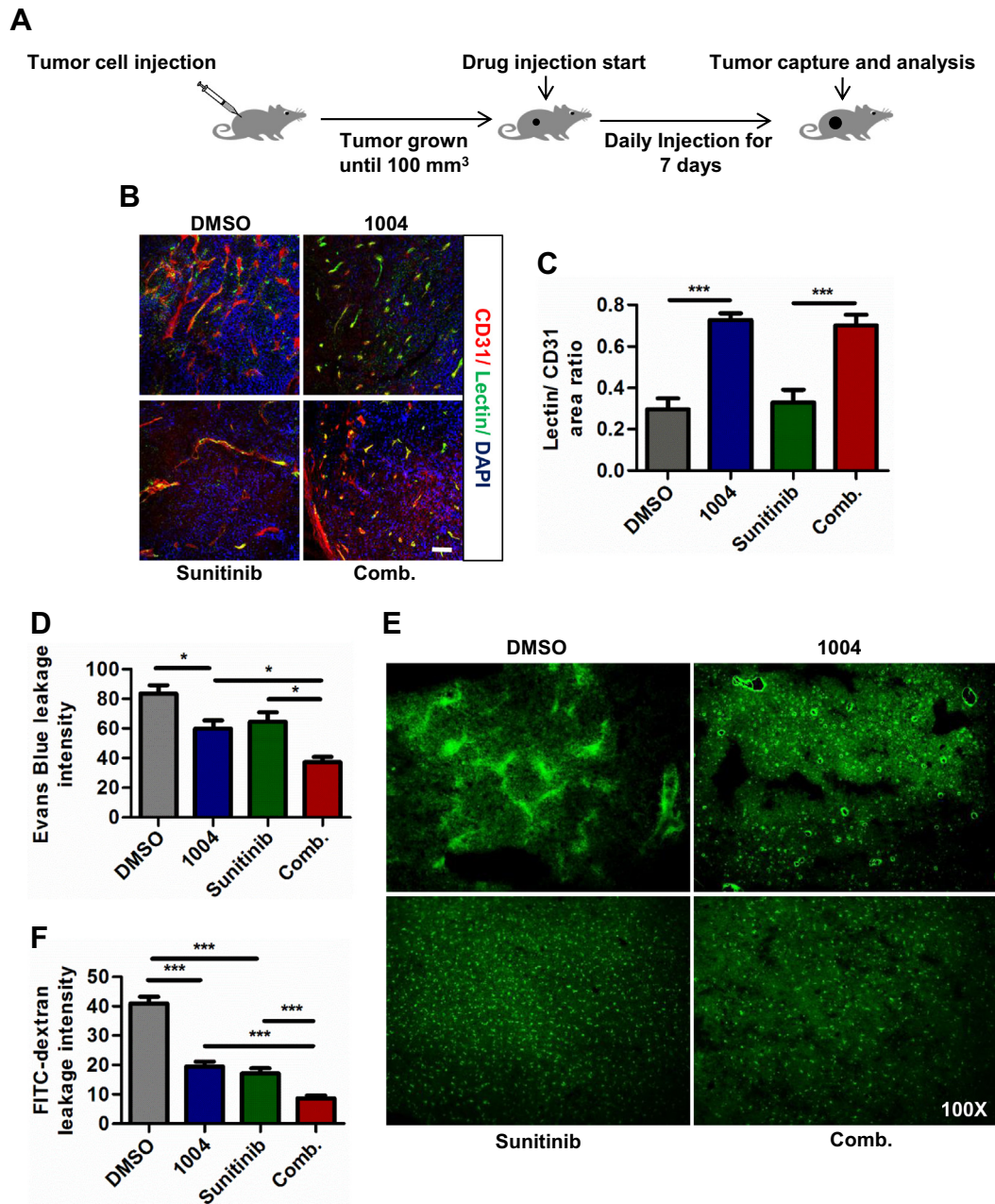


Fig. 2. Combination treatment of Sac-1004 and sunitinib significantly increases the ratio of functional blood vessels and decreases tumor vascular leakage. (A) Schematic plan for the administration of control (DMSO), Sac-1004, sunitinib or combination to tumor-bearing mice. (B) Biotinylated-lectin injected B16F10 tumors were sectioned and immunostained with CD31 antibody. Blood vessels double stained with CD31 and lectin represents functional one. Representative images from five different tumors (each group) has been shown. Scale bar, 100 μ m. (C) Quantitative assessment of lectin positive blood vessels from images shown in (B) using Multi Gauge program. (D) Vessel leakage in B16F10 tumor-bearing mice was accessed quantitatively using Evans blue dye method ($n = 5$). (E) Tumor vascular leakage in all 4 groups was visualized using FITC-dextran. Random pictures of three section per tumor ($n = 5$) were taken and representative pictures have been shown for comparison. (F) Images shown in (E) were quantified using ImageJ software. Data represent mean \pm SEM. * $P < 0.05$; *** $P < 0.001$.

combination treatment of Sac-1004 and sunitinib has a synergistic inhibitory effect on B16F10 tumor vessels hyperpermeability.

3.4. Sac-1004 increases pericyte coverage of blood vessels in sunitinib treated tumors

Coverage of blood vessels by pericytes is another crucial factor for the stabilization of tumor vessels and hence a determinant of vascular functionality. As we demonstrated previously [12], Sac-1004 treatment increased the number of pericyte covered blood vessels in B16F10 tumor (Fig. 3A and B). High magnification images

of blood vessels (inset) also demonstrated increased pericyte coverage of blood vessels (Fig. 3A). In contrast to Sac-1004 treatment, we found that sunitinib administration reduced the number of pericyte covered blood vessels, indicating that not only mature but also mature blood vessels are attacked by anti-angiogenic therapy. Upon co-treatment with Sac-1004, ratio of pericyte covered blood vessels was significantly increased as compared with sunitinib alone; however, no difference was observed between Sac-1004 and combination group (Fig. 3A and B). These results demonstrate that co-treatment of Sac-1004 and sunitinib is capable of normalizing tumor blood vessels.

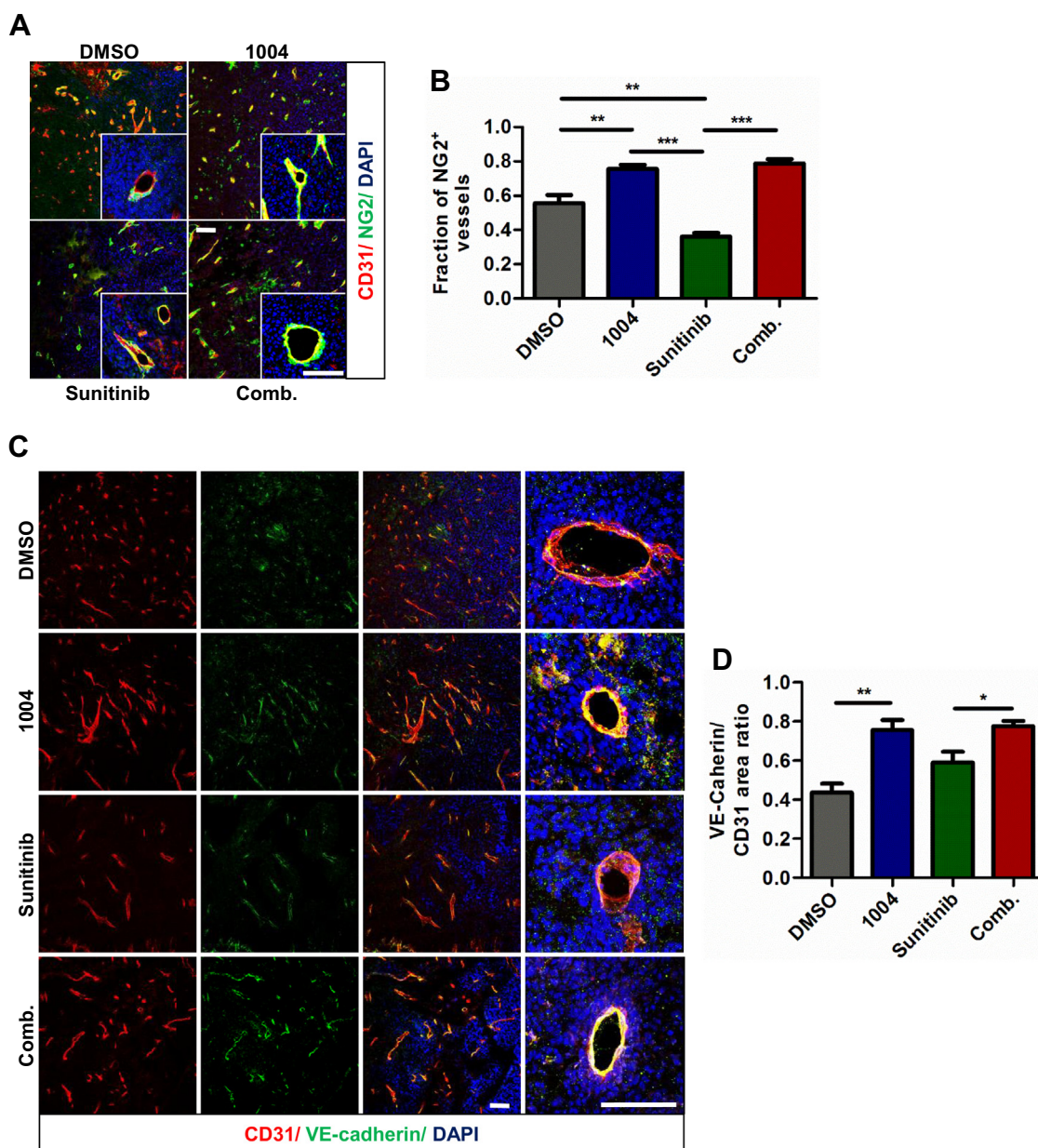


Fig. 3. Combination treatment of Sac-1004 and sunitinib significantly increases pericyte coverage and VE-cadherin content of tumor blood vessels. (A) Immunofluorescence staining of B16F10 tumor sections for blood vessels and pericytes. Low magnification images show fraction of blood vessels covered with pericytes. Extent of coverage is evident in high magnification images (inset). Representative images from five different tumors has been shown. Scale bar, 100 μ m. (B) Quantitative assessment of NG2 positive blood vessels. (C) B16F10 tumor sections were immunostained with CD31 and VE-cadherin antibody. Nuclear staining was done using DAPI. Low magnification images show the fraction of blood vessels covered with VE-cadherin, whereas high magnification images show VE-cadherin content in vessels of all four groups. Representative images from five different tumors (each group) has been shown. Scale bar = 100 μ m. (D) Images shown in (C) were quantified using Muti Gauge program. Data represent mean \pm SEM. * P < 0.05; ** P < 0.01; *** P < 0.001.

3.5. Sac-1004 enhances the sunitinib-induced strengthening of vascular junction in tumor

Various previous studies have suggested that enhanced vascular barrier could improve vascular structure and function [12,19,20]. Immunohistochemical analysis of B16F10 tumor sections showed that Sac-1004 treated group had increased number of VE-cadherin positive vessels (Fig. 3C and D), as noted previously [12]. Sunitinib treatment also increased the ratio of VE-cadherin positive vessels slightly; however, this increment was significantly enhanced upon combination with Sac-1004 (Fig. 3C and D). Also, examination of high magnification images of blood vessels revealed substantial increase in VE-cadherin content of blood vessels corresponding to combination treatment group as compared to that of sunitinib

group (Fig. 2A and B). However, no visible increase in VE-cadherin content was observed between Sac-1004 and combination group.

3.6. Combined treatment of Sac-1004 and sunitinib reduces vascular density and hypoxia in tumor

Antiangiogenic therapy, as with sunitinib, is associated with obliteration of immature blood vessels in tumor environment [8]. Here we found that Sac-1004 and sunitinib, both were able to reduce the blood vessel area of B16F10 tumor, though to different extents (Fig. 4A and B). Upon combination with Sac-1004, sunitinib treatment showed an increase in the area of blood vessels as compared with sunitinib alone, which was very similar to that in Sac-1004 group (Fig. 4A and B).

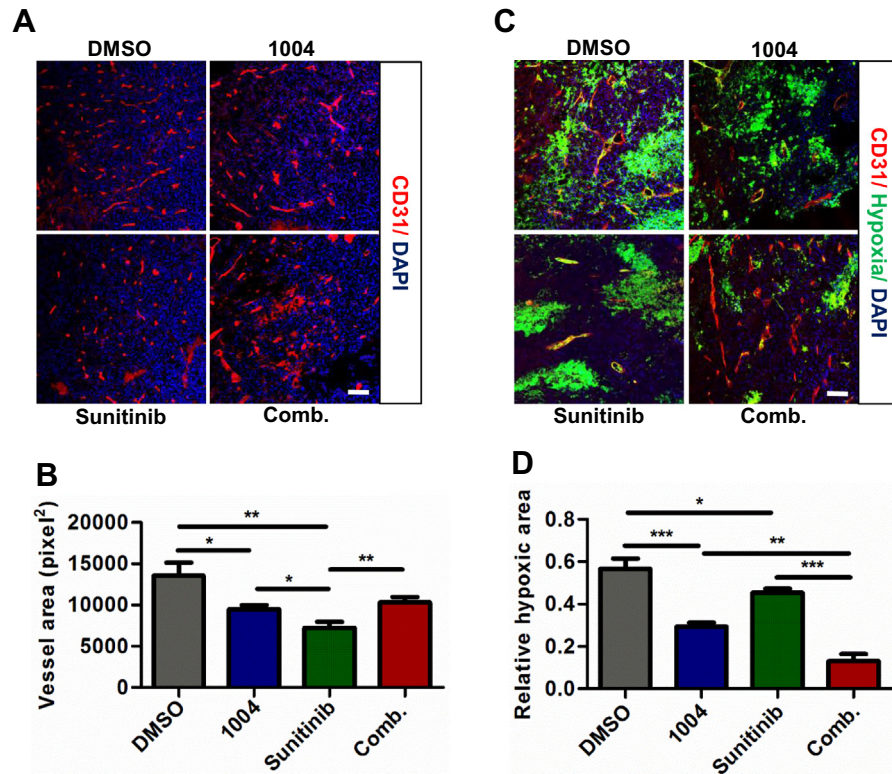


Fig. 4. Sunitinib reduces vascular coverage, but decreases hypoxia in combination with Sac-1004. (A) Immunofluorescence staining of B16F10 tumor sections with CD31 antibody. Representative images from five different tumors has been shown. Scale bar, 100 μ m. (B) Quantification of immunofluorescence images shown in (A) using Multi Gauge software. (C) Tumor sections from all four groups were immunostained for blood vessels and hypoxia (pimonidazole). Nuclear staining was done using DAPI. Representative images from five different tumors has been shown. Scale bar, 100 μ m. (D) Quantitative assessment of pimonidazole positive area. Data represent mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Reduction of vessel density is frequently associated with increase in hypoxia [21]. So we analyzed hypoxia in Sac-1004/sunitinib/combination treatment receiving tumors via pimonidazole staining. Sac-1004 treated tumors displayed reduced hypoxia (Fig. 4C and D), as noticed previously [12]. Sunitinib treatment also slightly reduced hypoxia; however, this reduction was much pronounced when sunitinib treatment was combined with Sac-1004 (Fig. 4C and D). These results suggest that combination therapy with Sac-1004 and sunitinib could help in soothing of tumor environment.

4. Discussion

In the present study, we have explored the strategic benefit of combining anti-angiogenic therapy with a vascular-hyperpermeability reducing agent on tumor therapy. Treatment of syngeneic tumor B16F10 with Sac-1004 and sunitinib combination therapy significantly decreased tumor growth and increased tumor-bearing mice survival. This strategy also substantially reduced hypoxia in tumor environment, which might be able to wane the aggressiveness of tumor cells and possibly reduce metastasis.

Sunitinib is a potent antiangiogenic agent, which have inhibitory activity against multiple tyrosine kinases such as vascular endothelial growth factor (VEGF)1, VEGFR2, VEGFR3, platelet derived growth factor receptor (PDGFR) α , PDGFR β , stem-cell growth factor receptor, fms-like tyrosine kinase (FLT)3, rearranged during transfection (RET) and colony-stimulating factor (CSF)1 receptor [22,23]. Given that above mentioned tyrosine kinases are crucial not only for angiogenesis but also for tumor cell survival, sunitinib exerts significant antiangiogenic and anti-tumor effect. Treatment of tumors with sunitinib initially targets imma-

ture blood vessels leaving behind mature ones, which results in improved functionality of tumor vessels [23–26]. This normalization effect is however, followed by a stage where due to continuous vessel obliteration, tumor cells face an adverse environment of increased acidity and hypoxia. Being educated in such an environment, tumor cells develop resistance to therapy and become more aggressive toward metastasis [11]. So we sought that if hypoxia development in tumor environment could be controlled by some means, antiangiogenic therapy as with sunitinib might have better therapeutic effects.

Recently, we found that a ginsenoside derivative Sac-1004 was potentially able to reinforce vascular junction and hence reduce tumor vascular hyperpermeability. Besides being able to normalize tumor vessels, Sac-1004 was able to decline tumor hypoxia substantially and sustainably [12]. Upon combination of sunitinib with Sac-1004, we found that hypoxia in B16F10 tumor environment decreased significantly as demonstrated by pimonidazole staining. Hypoxia is frequently associated with chemo-resistance, metastasis and tumor recurrence [10]. Indeed, sunitinib therapy is sometimes associated with increased tumor malignancy and metastasis [11]. Given the activity of Sac-1004 in delaying tumor progression toward malignancy and decreasing metastatic potential of tumor cells as was observed previously in MMTV and B16BL6 tumor [12], we speculate that combination therapy of sunitinib and Sac-1004 might be able to pacify tumor cell aggressiveness and metastatic potential. In accordance, combination therapy of sunitinib and Sac-1004 was able to reduce tumor growth and prolong the life-span of tumor-bearing mice.

Tumor vascular functionality is an important determinant of the effectiveness of cytotoxic therapy. Antiangiogenic therapy has been shown to improve vascular functions by reducing vascular

leakage, increasing vessel patency and by normalizing them, at least for a time window. This time window offers the benefit of enhanced cytotoxic therapy when administered in combination [9]. Sac-1004 was also shown to normalize tumor blood vessels and increase perfusion, sustainably [12]. In our study, combining sunitinib with Sac-1004 therapy reduced blood vascular leakage to an extent, which was much pronounced than either therapy alone. Also, sunitinib-induced vascular normalization was much improved by Sac-1004 co-treatment. Thus, it seems that combining vessel-diminishing therapy with vessel-stabilizing one could produce better functional vessels, leading to enhanced therapeutic outcome.

It has frequently been observed that antiangiogenic therapies are associated with certain undesirable side effects. Vascular obliterating activity of antiangiogenic molecules is not specifically confined to tumors, but affect normal vessels too. So long term or high dosage are also associated with an increased risk of arterial thromboembolic events, hypertension, renal side effects, and impaired wound healing [27,28]. Previously, we showed that Sac-1004 administration to mice did not show any visible sign of toxicity. Histological and biochemical tests also indicated that systemic administration of Sac-1004 might not be toxic to the animal [12]. In addition, as Sac-1004 was also shown to have protective effect (against apoptosis) on endothelial cells [14], we speculate that the combination therapy of sunitinib and Sac-1004 might not have the side-effects that were observed in case of sunitinib therapy alone.

In conclusion, here we have extended our study of tumor micro-environment-modulating activity of Sac-1004 and found that combining Sac-1004 with antiangiogenic therapy might improve the survival of tumor-bearing mice to a greater extent and possibly reduce tumor malignancy by curbing hypoxia.

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