**In vivo** spectral and fluorescence microscopy comparison of microvascular function after treatment with OXi4503, Sunitinib and their combination in Caki-2 tumors

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Abstract: Vascular targeting agents on their own have been shown to be insufficient for complete treatment of solid tumors, emphasizing the importance of studying the vascular effects of these drugs for their use with conventional therapies in the clinic. First-pass fluorescence imaging combined with hyperspectral imaging of hemoglobin saturation of microvessels in the murine dorsal window chamber model provides an easily implementable, low cost method to analyze tumor vascular response to these agents in real-time. In this study, the authors utilized these methods to spectroscopically demonstrate distinct vessel structure, blood flow and oxygenation changes in human Caki-2 renal cell carcinoma following treatment with OXi4503 alone, Sunitinib alone and both drugs together. We showed that treatment with OXi4503 plus Sunitinib destroyed existing tumor microvessels, inhibited blood vessel recovery and impaired Caki-2 tumor growth significantly more than either treatment alone.

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**OCIS codes:** (170.6280) Spectroscopy, fluorescence and luminescence; (170.2520) Fluorescence microscopy; (100.2960) Image analysis; (170.0180) Microscopy.

References and links

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1. Introduction

Various pathologies are characterized by the formation of abnormal vascular development. Tumor growth dependence on vascular formation and acquisition of adequate blood supply has led to significant effort towards the development of vascular targeting agents (VTAs) to treat solid tumors [1,2]. Two major classes of VTAs are vascular disrupting agents (VDAs) which act to destroy existing tumor vasculature by targeting their rapidly proliferating endothelial cells and angiogenic inhibiting agents (AIAs) that aim to inhibit the development of new tumor vasculature by targeting angiogenic stimulators and cytokines [3–9]. As single agents, VDAs and AIAs have been shown to be insufficient for complete treatment of solid tumors. VDAs leave a viable rim of tumor cells and vasculature from which tumors continue to grow, and AIAs have complications with developed resistances and potential increases in tumor hypoxia and metastatic burden [5,10–12]. Preclinical investigation into the use of these agents in combination showed enhanced tumor growth delay in comparison to either treatment alone, yet tumors still eventually grew to endpoint volumes [13,14].

The lack of evidence demonstrating VTAs as capable of constraining tumor growth on their own requires the administration of these agents in concert with conventional treatments such as radiation therapy for their use in the clinic. However, for the optimal use of VTAs as a concomitant therapy, extensive preclinical characterization of their effects on tumor microvasculature is necessary to determine which VTAs should be used and at what intervals, as changes in blood flow and oxygenation can have deleterious consequences on the therapeutic efficacy of conventional treatments.

There are many imaging modalities currently used for the evaluation of tumor microvasculature in the clinical setting. Positron emission tomography (PET) [15], computed tomography (CT) [16] and magnetic resonance imaging (MRI) [17,18] techniques are a few of the available methods that can be used in both the preclinical or clinical setting. With the aforementioned methods of analysis the primary clinical advantage is with non-invasive evaluation of deep tissue. In the preclinical setting, optical imaging methods such as transillumination and fluorescence microscopy provides a lower cost means of analysis while providing higher spatial and temporal resolution [19–21]. The mouse dorsal skinfold window chamber model offers the opportunity to use various forms of optical imaging to analyze the structure and function of tumor microvasculature at a microvessel spatial resolution.

We have developed the combination of widefield hyperspectral imaging microscopy of hemoglobin (Hb) saturation and first-pass fluorescence (FPF) imaging of blood transit time in a murine dorsal skinfold window chamber model as an inexpensive, easily implemented and valuable tool for preclinical analysis of oxygenation and blood flow through microvessel networks of tumors and their response to VTAs [22]. Previously, Wankhede et al. utilized Hb saturation imaging alone to investigate the oxygenation of tumor microvessels following treatment with OXi4503 [23]. While this analysis revealed microvessel functional changes via oxygenation data, no blood flow information was gathered to further characterize the treatment response. FPF imaging of blood transit time has been used previously to characterize the transient changes in blood flow in developing tumors over time by recording the flow of fluorescent contrast agents throughout the vasculature [22,24,25]. This addition of FPF imaging provides insight into changes in blood flow and vessel morphology by measuring the blood supply time (BST) of vessels in the tumor. The BST is defined as the time it takes for fluorescent tracers to travel to individual microvessels in a vascular network following injection relative to a reference supplying arteriole. In the present study, the combination of hyperspectral imaging of Hb saturation and FPF imaging of blood transit time is used to analyze the structural, blood flow and oxygenation changes that occur in Caki-2 human renal cell carcinoma (RCC) vasculature during treatment with the VDA OXi4503, AIA Sunitinib and both of these drugs in combination.
2. Materials and methods

2.1 Animal and tumor models

Caki-2 human RCC cells (a gift from Dr. Susan Knox, Stanford University) were cultured in vitro in DMEM (Cellgro, Herndon, VA) containing 10% fetal bovine serum (Bio-Whittaker, Inc) and 1% penicillin-streptomycin (Hyclone). Cells were incubated at 37°C in a 5% CO₂ atmosphere.

A titanium dorsal skinfold window chamber was surgically implanted onto female nude mice (NCR nu/nu) (>21g; Harlan Laboratories, Indianapolis, IN) under anesthesia with an intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg) [26]. Briefly, a 12 mm in diameter circular area of the upper layer of dorsal skin was removed and a single cell suspension (10 µL) of ~7.5x10⁴ tumor cells in phosphate buffered saline (PBS) was injected subcutaneously to generate a tumor ~4 mm³ in volume in 5-7 days. A round glass coverslip was placed over the exposed window. Animals were then housed in an environmental chamber at 33°C and 50% humidity with free access to food and water with a standard 12-hour light/dark cycle. All in vivo procedures were conducted under a protocol approved by University of Florida Institutional Animal Care and Use Committee.

2.2 Drug preparation and experimental design

OXi4503 (OXiGENE, Inc; San Francisco, CA) and Sunitinib (Pfizer, Pharmaceuticals; New York, NY) were given at a volume of 0.01 mL/g of the mouse body weight. OXi4503 (10 mg/kg) was given intraperitoneally in a solution of sterile saline and 50 µL/mL of 5% sodium carbonate. Sunitinib (100 mg/kg) was administered via oral gavage in a vehicle of citric acid monohydrate and sodium citrate dihydrate at a ratio of 1:16.3, adjusted to a pH of 3.5.

Mice were divided into four groups: untreated (control) (n = 6), OXi4503 alone (n = 5), Sunitinib alone (n = 7) and both agents together (n = 7). Once tumors reached a volume of ~4 mm³, experiments were initiated (day 1). Experiments were carried out for 7 days. Sunitinib was given on days 1-6 and OXi4503 on days 1, 3 and 5. Imaging was performed on days 1, 3, 5 and 7 immediately before administration of any drugs.

The length, width and height of each tumor nodule protruding from the back side of the window chamber were measured daily using calipers (Fig. 1). These dimensions were used to calculate the volume of the tumor in mm³ using the equation for half of an ellipsoid.

\[
\text{Tumor volume} = \frac{1}{2} \pi \times \text{length} \times \text{width} \times \text{height}
\]

Fig. 1. Tumor volume measurement. (a) Front view of an installed window chamber with tumor outlined in black. (b) Back side of window chamber with tumor nodule outlined. The red lines indicate the measured length and width of the tumor nodule. (c) The height is measured as the distance the tumor nodule protrudes from the back of the window chamber, indicated by the red line.

2.3 Fluorescent liposome preparation

Liposomes were prepared using the lipid hydration method [27]. All lipids and extruder were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Liposomes were composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine
DOPS), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPC-mPEG2000) and cholesterol combined at a molar ratio of 47.4:19.7:1:39.7. Liposomes were fluorescently labeled with dye in ethanol (2 mM) added at 0.5 to 1 mol.% of the total phospholipid content. Fluorescent carbocyanine dyes 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate (DiD; ex 644 nm/em 665 nm) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR; ex 750 nm/em 780 nm) were purchased from Invitrogen (Carlsbad, CA). This mixture was dried into a thin film with nitrogen gas and held under vacuum overnight to remove traces of solvent. The film was rehydrated in sterile PBS (pH = 7.4) to yield a suspension of multilamellar vesicles at a lipid concentration of 10 mg/mL. Unilamellar liposomes ~100 nm in diameter were obtained by repeated extrusion through a Nuclepore membrane (100 nm pore size).

2.4 Intravital microscopy

Brightfield images were taken daily to monitor changes in tumor vasculature morphology. Hyperspectral imaging of Hb saturation was completed first followed by FPF imaging. A Zeiss AxioImager microscope (Carl Zeiss, Inc., Thornwood, NY) with a 2.5x fluor long working distance objective (Carl Zeiss, Inc., Thornwood, NY) was used as the imaging platform. Mice were anesthetized using 1.0-1.5% isoflurane in air (smallest dose for immobilization used as this minimized anesthesia effects on HbSat) and placed on a heated stage.

2.5 Hb saturation imaging and analysis

Hyperspectral imaging of microvessel Hb saturation (Fig. 2) was described in detail previously [23,26]. Briefly, a 100 W tungsten halogen lamp was used for transillumination of the window chamber. A liquid crystal tunable filter (400-720 nm; CRI, Cambridge, MA) and a monochromatic scientific grade charge coupled device (CCD) camera (DVC Company, Austin, TX) thermoelectrically cooled to −20°C was used to acquire spectral image data sets from 500 nm to 575 nm at 5 nm intervals. The final field of view was 4.15 mm in width by 3.1 mm in height. Pseudocolor maps of Hb saturation were created in MATLAB® (The Mathworks Inc., Natick MA) using pure oxy- and deoxyhemoglobin reference spectra and a previously determined linear least squares regression model [28]. Hb saturation maps were cropped in width to correspond to the same field of view as BST maps.

2.6 FPF imaging and analysis

FPF imaging of blood transit time was discussed in detail previously (Fig. 3) [22]. Fluorescence images were acquired using an ANDOR iXon electron multiplying CCD camera (ANDOR Technology, South Windsor, CT) thermoelectrically cooled to −50°C. The final field of view was 3.1 mm in width by 3.1 mm in height. An injection of ~50 µL of fluorescent liposomes was administered via tail vein injection and 700 stacked TIFF images
were simultaneously acquired at a total frame exposure time of 0.117 s to capture the transit of liposomes throughout the entire vascular network. DiD and DiR liposomes were alternated daily to allow for sufficient clearance of liposomes from the field of view. Cy5 (ex 640 ± 20 nm / em 680 ± 30 nm) and Cy7 (ex 740 ± 35 nm / em 795 ± 50 nm) filters (Chroma Technology Corp., Rockingham, VT) were used to capture fluorescence from the DiD and DiR labeled liposomes respectively.

BST maps were created using MATLAB® and MeVisLab (MeVis Medical Solutions AG, Bremen, Germany) software. A vascular mask was created for each image stack in MeVisLab using thresholding, Gaussian smoothing, Hessian vesselness and fuzzy c-means algorithms. In MATLAB®, each image stack was truncated to the frame at which liposomes first entered the field of view and the frame at which the liposomes completely filled the microvasculature. To create a BST map, every pixel in the mask was designated a BST based on its fluorescence intensity profile over time. The value was determined as the time at which the pixel signal reached its average intensity relative to the arteriole in which fluorescence flow was first detected. Vessels that were more rapidly filled with liposomes registered faster BSTs and vessels filled at a later time registered slower BSTs. Region of interest analysis was performed to correlate BST and Hb saturation in 30 specific vessel areas and monitored over time. Vessel density for each day was calculated from BST maps by dividing the number of vessel pixels by the total number of pixels in the visible tumor area.

Fig. 3. First-pass fluorescence imaging. Fluorescent liposomes are injected and simultaneously recorded as the liposomes first enter the field of view. Frame (1) represents ~1.9 s after injection of liposomes, (2) ~5.8 s, (3) ~7.7 s, (4) ~11.6 s, (5) ~19.3 s and (6) ~30.8 s. MATLAB® is then used to create a vascular mask of the vessel area and assign BSTs to each pixel in the vascular mask. The BST is defined as the time it takes to reach the average pixel intensity for each individual pixel in the vascular mask. In the final BST map, red corresponds to faster BSTs and blue longer BSTs.

2.7 Immunohistochemical analysis of window chamber tumors

Following image acquisition on day 7, mice were euthanized and tumors were harvested from window chambers. Tumors were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, USA, Inc., Torrance, CA), frozen and stored at −80°C. Acetone-fixed cryosections (5 µm) were immunolabeled using rat anti-mouse endothelial cell antigen (MECA)-32 (1:500, BD Pharmingen, San Diego, CA), an endothelial cell marker, then donkey anti-rat Alexa Fluor® 594 (1:500). Tissue sections were cover slipped using aqueous mounting media containing 4’-6-diamidino-2-phenylindole (DAPI) (Vector Shield, Vector Laboratories Inc., Burlingame CA).

2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Tumor volumes, immunohistochemical (IHC) fluorescence signal intensities and average BST, Hb saturation and vessel densities over time were tested using a nonparametric, two-tailed Mann-Whitney rank sum test. Results with p<0.05 were considered significant.
3. Results

3.1 Tumor growth inhibition

Figure 4 shows that all treatment groups had statistically significant tumor growth inhibition compared to control on day 7 (p<0.01). With respect to controls, final tumor volumes were inhibited 4.8 fold in the OXi4503 group, 5.8 fold in the Sunitinib group and 13.8 fold in the combination group. Combination treatment significantly arrested tumor volume in comparison to OXi4503 (p<0.01) and Sunitinib (p<0.001) treatment groups on day 7. There was no significant tumor growth throughout the experiment in combination treated mice as well as no significant difference between Sunitinib only and OXi4503 only tumor growth by day 7.

![Fig. 4. Daily tumor volume measurements of tumor nodules in window chambers (median ± interquartile range). All treatment groups showed tumor growth delay compared to controls (p<0.01), but combination treatment showed significantly greater tumor volume suppression in comparison to the OXi4503 (p<0.01) and Sunitinib (p<0.001) single agent groups.]

3.2 Morphological changes of tumor blood vessels

Daily brightfield images revealed changes in tumor vessel morphology for each experimental group (Fig. 5). Control tumors exhibited an increase in microvessel density by day 7. Individual vessels became more tortuous and varied in thickness. It was difficult to identify the same vessel structures daily, an indication of rapid vessel development in growing tumors.

Analysis of the central tumor area showed that OXi4503 treated tumors displayed a characteristic pattern of tumor vessel destruction from the core out to the rim of the tumor following initial treatment. After 48 hours, on day 3, little vessel recovery in the periphery was seen. After repeated treatments we observed less vessel damage and more aggressive vessel recovery directed from the periphery towards the tumor core. The vessels that recovered and developed into the tumor core were similar in appearance to those of control tumors.

Tumors of Sunitinib treated mice displayed a decrease in microvessel density over time. The major vessel structures of the tumors were maintained throughout the experiment with smaller vessels being pruned.

Combination treatment resulted in vascular destruction in the tumor core akin to tumors treated with OXi4503 alone. However, there was no vessel regrowth back into the tumor core over the course of treatment. In contrast to vessels in the tumor, normal vessels surrounding the tumor maintained their structures.
3.3 Immunohistochemical analysis

Tumor slices were stained with MECA-32 for endothelial cells and DAPI for nuclear material. Figure 6(a) shows examples of merged staining images for each experimental group. The vessels of control tumors were in general much larger and occurred at a higher density in comparison to tumors in the three treatment groups. Although OXi4503 treated tumors had a higher presence of MECA-32 staining, the vessels in these tumors were fractured rendering their function as debatable. Sunitinib treated tumors had much smaller vessels with a lower density in comparison to control and OXi4503 treated tumors. Although vessel density and size were similar between combination and Sunitinib treated tumors, the functionality of
combination treated vessels was highly suspect given observations from imaging analyses (Fig. 7).

Figure 6(b) shows the ratio of MECA-32 to DAPI staining for 10 random regions in tumors from the control (n = 5), OXi4503 alone (n = 4), Sunitinib alone (n = 4) and combination treated tumors (n = 4). The MECA-32 stain was normalized to the amount of nuclear material to account for variations in cellular density. All treatment groups demonstrated a significantly lower MECA-32 to DAPI ratio in comparison to controls (p<0.001) indicating less endothelial cell staining. However, without a histologic marker of perfused microvessels (e.g., Hoechst 33342 [29]), this metric alone has limited value. As shown in the next section, the in vivo functional imaging yields additional information not available in histology images.

3.4 FPF and Hb saturation imaging of blood flow and oxygenation

Whole image Hb saturation, BST and vessel density averages for each treatment group are shown in Fig. 7 and plots of Hb saturation vs. BST are shown in Fig. 8 for the thirty regions of interest from the images in Fig. 7. In normal blood flow, faster BSTs generally correspond to higher oxygenation values and slower BSTs correspond to lower oxygenation values [21, 22]. As shown in Fig. 8, for tumor microvasculature this relationship can become less well defined.
Fig. 7. Hb saturation and BST maps over time. In control tumors, BSTs became longer and oxygenation decreased as complicated vessel pathways formed. OXi4503 treated tumors displayed vascular damage up to the rim of the tumor after initial treatment, resulting in neovascuature in the tumor periphery with faster BSTs and a higher oxygenation (day 3). Each subsequent treatment was less effective, leaving larger vascular rims with greater variation in BSTs and oxygenation, resembling that of control tumor vessels. Sunitinib treated tumor vessels exhibited faster, more homogenous BSTs with higher oxygenation values that were maintained. The combination treatment left no tumor vessels to be analyzed.
Fig. 8. Scatter plots correlating the Hb saturation and BST value of 30 specific vessel regions of interest in tumors shown in Fig. 7. The control tumor resulted in longer BSTs and lower Hb saturation values. OXi4503 suppressed this trend initially on day 3, but began to develop BST and Hb saturation values similar to the control tumor by day 7. The Sunitinib treated tumor showed inhibition of long blood supply times and maintenance of Hb saturation values. It is important to note that the values associated with the combination treated tumor correspond to normal vessels surrounding the tumor since all detectable tumor vessels were eradicated.

FPF and Hb saturation imaging in Fig. 7 together with averages of Hb saturation, BST, and vessel density in visible tumor areas in Fig. 9 revealed differences in blood flow and oxygenation effects for each experimental group. Control tumors overall developed statistically significantly (p<0.05) increased BSTs and decreased oxygenation (lower Hb saturation) with time. The increase in BST heterogeneity over time was indicative of increasingly chaotic microvessel morphology as liposomes, and thus blood, required longer times to travel to specific tumor areas.
In OXi4503 treated mice, tumor vessels were destroyed up to the rim of the tumor after initial treatment, leaving behind a well oxygenated rim of vessels with fast BSTs. Over time new vessels formed from the rim, originating from surrounding normal vessels and surviving peripheral vessels with faster BSTs and a higher overall oxygenation throughout. With repeated treatments, tumor vessels in areas of regrowth exhibited less damage with more recovery into the tumor core and started to resemble control tumor vessels in terms of BST, oxygenation, and vessel density as tumors overcame the effects of OXi4503 treatment.

Tumors in the Sunitinib treatment group developed an increase in oxygenation and decrease in BST. Along with faster BST values, the variability in BSTs decreased as larger vessel structures were retained. The homogeneity in BSTs indicated that Sunitinib treatment had resulted in less convoluted or obstructed blood flow pathways.

Combination treated tumors left no visible tumor vessels to be analyzed. The surrounding normal vessels were maintained around the periphery of the tumor until day 7. On day 7, faster blood supply times and higher oxygenation values were observed, indicating the formation of new vessels in the tumor periphery. Statistically, the combination treatment had the lowest vessel density (p<0.05).

It should be noted that even more detailed image analysis than that presented here may reveal even more information. For example, although the analysis of the visible tumor area average Hb saturation, BST and vessel densities at day 7 for OXi4503 and Sunitinib single agent treatments are statistically similar, the images in Fig. 7 suggest that a subregional analysis would uncover more subtle differences in these parameters.

4. Discussion

An advantage of the dorsal skinfold window chamber model is the ability to simultaneously assess microvascular development and tumor growth as a function of time. There are many optical imaging modalities available to characterize different aspects of vascular function and development in the window chamber model. Fluorescence recovery after photobleaching (FRAP) [30], multiphoton microscopy [31] and laser scanning confocal microscopy [32] have
been utilized to characterize vessel leakiness and permeability in wound healing and growing tumors. With respect to angiogenesis, in addition to fluorescence and brightfield microscopy, new instrumentation and algorithms have been developed to apply optical coherence tomography (OCT) [33] for the high resolution analysis of vessel structure and development in tumors. While much can be derived from optical measurements of vessel structure, the ability to characterize both structural and functional aspects of vessel development in a single imaging platform is a great advantage for preclinical vascular research. Our combination of hyperspectral imaging of Hb saturation and FPF imaging of blood transit time not only allows for widefield analysis of tumor vessel structure at a microvessel spatial resolution, but also contributes functional analysis of oxygen transport and blood flow.

Multimodal measurement of microvascular oxygen transport function has been accomplished through the combination of several different optical techniques with spectral imaging of Hb saturation. For example, laser speckle imaging of blood flow [34,35], OCT measurement of blood velocity and flow direction [36], fluorescence imaging of red cell flux for convective oxygen mass transport [37] and video microscopy image processing to measure blood velocity and flow direction [38] have all been combined with spectral imaging of Hb saturation for the study of microvessel networks. The FPF imaging method employed in this study provides unique information that can complement information from the previously mentioned techniques. The BST measurement, a ratio equal to the blood flow pathlength divided by the average blood velocity between two vessels, shows microvessel network connections relative to a chosen supplying arteriole, thus yielding information on microvessel network shunting, remodeling of vessel connections and regional blood supply differences throughout a microvessel network. This information is not readily available from the aforementioned techniques, and can complement their information, particularly those methods that give true blood flow velocity.

Transillumination microscopy of tumors in the window chamber model has previously been used to show the vessel inhibition effects of Sunitinib in comparison to controls [39] as well as the time course by which tumors respond to the VDA Combretastatin A4 produg [40]. Furthermore, hyperspectral imaging of Hb saturation has been used previously to demonstrate the change in oxygenation of vessels in response to OXi4503 [23]. However, the aforementioned studies gave no data regarding tumor blood flow and vessel function. Our dual imaging approach revealed significant structural and functional differences in tumor microvasculature of control tumors as well as tumors of mice treated with the VDA, AIA or their combination. Our observations of increased microvessel densities, and trends towards longer BSTs and areas of lower oxygenation in untreated control tumors are consistent with previous studies of tumor growth [41,42]. More complicated vascular pathways formed in the growing tumor may lead to longer and more heterogeneous BSTs which are a potential cause of lower oxygenation in the tumor vessels (this correlation is shown in Fig. 8). In contrast to control tumors, measurements made on tumors of mice undergoing vascular targeting therapies showed that all treatments hindered the development of longer BSTs and lower oxygenation. While an inherent limitation to the analysis of tumors in the window chamber model is the restriction of tumor size, similar enhanced antitumor efficacy was observed when applying VDA plus AIA therapy to established larger Caki-1 human RCC [13,43], AML [44] and mouse PC3 prostate cancer xenografts [45].

The initial treatment response of Caki-2 tumors to OXi4503 that we observed is similar to previous reports [46–49]. Our study suggests that Caki-2 tumor vasculature recovery may overcome OXi4503 treatment efficacy with time, but previous studies from our laboratory have shown that the structural recovery of tumors following serial treatments of OXi4503 can vary depending on tumor type [23]. In contrast to our observations where Caki-2 tumors were able to overcome OXi4503 treatment, macroscopic KHT sarcomas previously treated with a VDA (Combretastatin or OXi4503) were found to be equally susceptible to repeat treatments with these agents [46]. Clearly such factors need to be taken into consideration in the clinical
application of these agents when used alone or in combination. In the context of combining VDAs with radiotherapy, when considering repeat treatments of OXi4503, our imaging results suggest that doses every 48 hours can result in more aggressive vascular development and the formation of less oxygenated regions in the vessel rim, posing a problem for effective treatment of a solid tumor.

Sunitinib can completely inhibit angiogenesis when administered simultaneously with tumor cell injections in mice bearing window chambers [39]. The effects of Sunitinib and other AIAs are less clear when dealing with existing tumor vasculature [12,50]. When compared to the untreated control tumor microvasculature, our imaging analysis of Sunitinib treatment supports the theory of vascular normalization [3] in terms of microvessel structure and oxygen transport function, at least over the time scale of our imaging experiments. Similar to our results, faster tumor blood flow and increased tumor perfusion following treatment with Sunitinib has been observed [51,52]. In addition, a window of normalization has also been reported in macroscopic tumor studies [41,50,53,54]. However, this window was transient and reversal of the benefits to anti-angiogenic therapy have also been observed [3,50]. It should be noted that despite more normalized microvessels, tissue hypoxia may still be present if oxygen supply is not adequate to meet local demand [55].

The combined treatment with both agents resulted in the most effective means of controlling the formation and proliferation of tumor microvessels. The characteristic vascular damage attributed to OXi4503 was complemented by the action of Sunitinib, which inhibited neovascularization, and the combined treatment led to necrosis in the tumor core with no functional vessels observed in the tumor interior. Although the IHC analysis indicated the presence of vessels and endothelial cells in the tumor interior, perfusion of fluorescent liposomes during FPF imaging was only observed in normal vessels around the tumor periphery. It is likely that regrowth observed on day 7 at the tumor rim is supported by normal tissue vessels that provide oxygen and nutrients necessary for the tumor cells to proliferate [49]. This observation supports our belief that even combination VDAs plus AIAs treatments should still be administered in conjunction with conventional therapies that can effectively eradicate any remaining tumor cells. However, such treatment strategies will require detailed knowledge of optimal timing of agents to maximize antitumor effects while minimizing potential toxicities [14,56]. As a promising start, studies involving the use of Combretastatin, Bevacizumab and conventional chemotherapies have shown that the addition of Combretastatin to each therapy was well tolerated with enhanced tumor effects in comparison to treatments without the use of the VDA [57,58].

In summary, we have demonstrated the use of the mouse dorsal skinfold window chamber model and the combination of hyperspectral imaging of Hb saturation and FPF imaging of blood transit time to measure the longitudinal response of Caki-2 tumors to OXi4503 and Sunitinib used either alone or in combination. Because mice bearing dorsal skinfold window chambers have the capability of being treated with both systemic therapies and radiation therapy, our combination imaging modality can be applied further towards the characterization and optimization of VTAs in concert with conventional therapies, emphasizing the utility of our combination imaging methodology as a valuable tool for evaluating vascular response to treatment in vivo.

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