Alterations in vascular architecture and permeability following OXi4503 treatment

Lie Sam Chan, Cathy Malcontenti-Wilson, Vijayaragavan Muralidharan and Christopher Christophi

OXi4503 retards tumor growth in a dose-dependent manner and improves survival in a murine model of colorectal liver metastases. This agent causes extensive vascular shutdown by selectively altering the tubulin cytoskeleton within the endothelial cells of tumor vessels. The destruction of tumor vessels is incomplete, however, and tumor revascularization occurs after the treatment. This study evaluates the pattern of microcirculatory changes and alterations to the ultrastructural properties of the tumor vasculature that result from OXi4503 treatment. Male CBA mice were induced with liver metastases via an intrasplenic injection of a murine-derived colorectal cell line. After administering a single intraperitoneal dose of OXi4503, changes in tumor perfusion, microvascular architecture and permeability were assessed at various time points. One hour after a 100-mg/kg dose of OXi4503, a significant decrease in the percentage of tumor perfusion (63.96 ± 1.98 in controls versus 43.77 ± 2.71 in treated mice, $P < 0.001$) was observed, which was still evident 5 days after the treatment. Substantial tumor microvascular damage and minimal normal liver injury were observed. Tumor vascular permeability was significantly elevated 45 min after the OXi4503 treatment (67.5 ± 3.60 in controls versus 80.5 ± 2.24 μg/g, $P < 0.05$). The findings suggest that OXi4503 selectively targets tumor vessels and causes immediate microvascular destruction. Even at the maximum tolerated dose, however, residual patent tumor vessels were still present after treatment, implying incomplete tumor destruction. A combination of OXi4503 with other chemotherapeutic modalities might achieve complete tumor eradication and improve long-term survival. *Anti-Cancer Drugs* **19:17–22 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

An intact blood supply is crucial in sustaining the continuous growth and survival of a tumor. Unlike antiangiogenic agents, which suppress the formation of tumor vessels, vascular-disrupting agents (VDAs) are a group of compounds that specifically target the mature tumor vasculature, occlude blood flow and cause tumor necrosis [1].

The combretastatins are a family of VDAs showing much clinical promise. The leading member from this family is combretastatin A4 phosphate (CA4P), which is currently undergoing phase II clinical trials. Studies have shown CA4P to be highly tumor vessel-specific and to cause immediate vascular shutdown at doses well below its maximum tolerated dose (MTD) [2]. Despite this, growth retardation was not observed because of the regrowth of the viable tumor rim that is less susceptible to the antivascular effects of CA4P [3,4]. Another member of the combretastatin family is OXi4503, which behaves in a similar manner to CA4P [5]. Preclinical studies have, however, shown this agent to have greater potency and, probably, some cytotoxic activity, in addition to its vascular-disrupting effects [6–8]. The enhanced cytotoxic activity can be attributed to its being metabolized *in vivo* into a reactive and cytotoxic o-quinone [9].

We have previously shown OXi4503 to significantly reduce tumor growth and prolong survival in a murine model of colorectal (CRC) liver metastases in a dose-dependent manner [10]. Several studies have demonstrated a rapid reduction in blood flow after treatment with OXi4503 in a variety of tumor models: this is attributed to an immediate disruption in the tumor microvasculature [11–13]. Despite causing significant vascular damage, previous studies have shown that vessel destruction is incomplete, which allows for tumor recurrence [11–14]. The pattern of vascular destruction and the ultrastructural changes after treatment with OXi4503 are uncertain.

The aim of this study is to investigate the alterations in tumor vasculature after treatment with OXi4503. Using a previously established murine model of CRC liver

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metastases, the changes in blood flow, and in vascular architecture and permeability were assessed after a single MTD of OXi4503.

Materials and methods

Animals

Male CBA mice, 6–8 weeks old (Laboratory Animal services, University of Adelaide, South Australia), were used in all the experiments. They were maintained in standard cages with access to food and water *ad libitum*. All procedures performed were implemented in accordance with the guidelines of the Austin Hospital Animal Ethics Committee.

Anesthesia and analgesia

Mice were anesthetized by an intraperitoneal injection of a freshly prepared mix of ketamine (100 mg/kg; Pfizer, New Zealand) and xylazine (10 mg/kg; Troy Laboratories, Australia). Carprofen (Pfizer, New Zealand) at a dose of 5 mg/kg was administered subcutaneously for analgesia. At the conclusion of each assessment, mice were killed via exsanguination.

Experimental model of colorectal liver metastases

CRC liver metastases were induced via an intrasplenic induction of a dimethyl hydrazine-induced primary colon carcinoma (50 000 cells/ml) in the CBA mouse using a previously established technique [15]. This model of liver metastases follows a Gompertzian growth pattern and closely resembles the human disease. Liver metastases are evident, and are vascularized by day 16 of the tumor inductions and are fully established by day 21.

Experimental groups

OXi4503 (combretastatin A-1 *trans*-stilbene), which had kindly been provided by Oxigene, was protected from light and suspended in sterile saline before injection. Sixteen days after tumor induction, the mice were administered an intraperitoneal injection of OXi4503 at 100 mg/kg. This dose had previously been established to be the single MTD in this experimental model of liver metastases [16]. At this dose, the CBA mice did not show signs of toxicity. Control groups received an equivalent volume of sterile saline.

Assessment of blood flow using laser Doppler flowmetry

Relative tumor blood flow was examined on a selection of tumors visible on the superficial surface of the liver. Perfusion measurements were taken with an Oxyflo 2000 Blood Perfusion Monitor (Oxford Optronix, Oxford, UK) using a previously described technique [15]. After performing a laparotomy on anesthetized mice, two laser probes were placed on a tumor and on an area of normal liver, respectively. The concurrent movement of the probe with the rate of breathing ensured that the probe was in contact with the tissue surface. Mean tumor perfusion was expressed as the percentage of tumor blood flow relative to the blood flow in the normal liver. Six animals/treatment group were used and a minimum of 20 tumors was assessed in each group.

Microvascular resin casting and scanning electron microscopy to examine microvascular architecture

Microvascular resin casts of the tumor and the normal liver were prepared as previously described [17]. A thoracotomy was performed on anesthetized mice. After the cannulation of the thoracic aorta, the vascular system was flushed using heparinized saline. This was followed by the injection of an acrylic resin, which was subsequently allowed to polymerize overnight. The casts were then prepared for viewing using a scanning electron microscope (Philips XL30 field emission scanning electron microscope, School of Botany, University of Melbourne, Australia). A total of six animals in each treatment group were used and a minimum of 10 tumors was examined.

Measurement of vascular permeability using Evans blue

Immediately after treatment with OXi4503, Evans blue (Sigma-Aldrich, Sydney, New South Wales, Australia) was administered intravenously at a dose of 30 mg/kg. Tumor tissue (200 mg) was placed separately into Eppendorf tubes with 2 ml of formamide. After homogenization had been carried out, the sample was then incubated for 48 h at 60°C for dye extraction. After centrifugation at a speed of 12 000 rpm for 20 min, the supernatant of the suspension was transferred into a cuvette for spectrum analysis at an absorbance of 620 nm. The amount of Evans blue (μg/g tissue) was calculated from standard solutions with the individual sample background being calibrated by the method of standard addition. A total of five animals were used at each time point and a minimum of three tumor samples were taken from each mouse.

Assessing real time changes in permeability patterns using in-vivo confocal microscopy

The acute effect of OXi4503 on the liver microvasculature was assessed by in-vivo confocal microscopy. At various time points after the OXi4503 treatment, 0.05 ml of fluorescein isothiocyanate dextrate (molecular weight 160 000) (Sigma-Aldrich) was administered intravenously followed by a laparotomy on the anesthetized mice to expose the liver surface. Mice were subsequently placed onto a viewing stage and examined under an Olympus BH2 microscope connected to an F990e personal confocal system (Optiscan, Victoria, Australia). Regions of altered permeability and microvascular injury were observed at various magnifications, and continuous recordings were taken over a period of 1 h.

Statistical analysis

All data are represented as mean ± standard error of the mean. Statistical analysis was conducted using SPSS (Statistical Package for the Social Sciences, version 10,

Chicago, Illinois, USA) using both parametric (analysis of variance) and nonparametric (Kruskall–Wallis) analytical tests as appropriate. All statistical tests were two-sided and $P < 0.05$ was considered to be statistically significant.

Results

Study 1: changes in tumor perfusion after treatment with OXi4503

Changes in tumor perfusion were examined 1 h and 5 days, respectively, after treatment with OXi4503. Acute reductions in perfusion were observed with tumor blood flow plummeting to 70% of control blood flow (63.96 ± 1.98 in controls versus $43.77\% \pm 2.71$; $P < 0.001$; Tukey HSD). This reduction in perfusion was maintained for 5 days after the OXi4503 treatment (68.43 ± 2.62 in controls versus $37.16\% \pm 1.62$; $P < 0.001$; Tukey HSD) (Fig. 1).

Study 2: alterations in vascular architecture

Alterations in the vascular architecture were assessed at the same time points as applicable to the blood flow measurements. Electron microscope scans of the normal liver showed a highly organized microvascular structure with regular-shaped and regular-sized vessels (Fig. 2a). No filling defects were observed, indicating patent vessels. This is in contrast to the tumor vasculature, which displayed spatially tortuous and disorganized vessels of varying shapes and sizes (Fig. 2b). Vessels were dilated and discontinuous with blind endings, and large vascular lakes were formed by the flattened and coalesced vessels. A direct sinusoidal supply into the liver metastases was indicated by the vascular continuity at the tumor host interface. In the magnified view of the tumor vasculature, small openings were observed within the endothelial wall, which were indicative of highly permeable tumor vessels.

Examination of the adjacent normal sinusoids found no damage caused by OXi4503 (Fig. 2c, *inset*). One hour after the OXi4503 treatment, substantial vascular destruction was observed (Fig. 2c). Vessels displayed a flattened morphology with gradual tapering and constriction toward regions of complete vascular occlusion. Filling defects were identified as an absence of tumor vessels or areas of necrosis. Complete vascular destruction was noted at the tumor host interface resulting in the formation of a halo at the tumor margin.

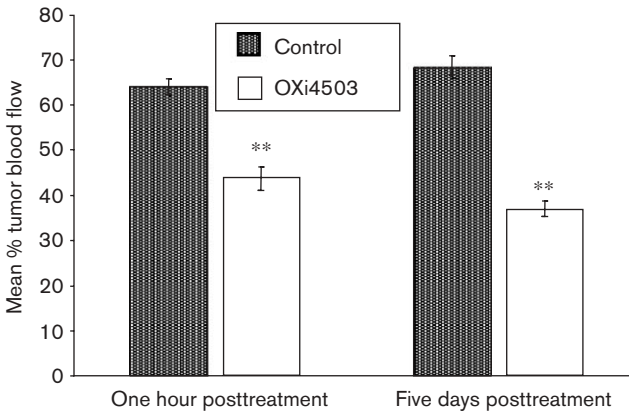
Five days after the OXi4503 treatment, regeneration of the tumor vasculature had occurred (Fig. 2d). Large vascular lakes were observed with filling defects located in the tumor center, indicative of central necrosis.

Study 3: changes in vascular permeability

Changes in the vascular permeability after the OXi4503 treatment were assessed at 15-min intervals. After the administration of the fluorescent dye, normal liver hepatocytes exhibited maximum and homogenous fluorescence intensity with minimal dye leakage (Fig. 3a). Conversely, the tumor vasculature displayed reduced fluorescence intensity indicating highly permeable vessels, which accounts for the dye leakage. The intensity within the tumor is heterogenous, with some areas showing greater fluorescence intensity (Fig. 3b). Fifteen minutes after the OXi4503 treatment, an immediate reduction in the fluorescent intensity was observed (Fig. 3c). As time progressed, fewer vessels were visible; the fluorescence was minimal 60 min after the treatment (Fig. 3d–f).

Despite the changes in fluorescence observed in the confocal images, assessment of permeability using an Evans blue assay found conflicting results (Fig. 4). Unlike the control group, which showed no significant fluctuations in permeability, the OXi4503-treated group displayed a progressive increase in permeability, which was maximal at the 45-min time point (67.50 in controls versus 80.50 ± 2.24 μg/g, $P < 0.01$; Tukey HSD). The elevation in permeability had returned to the levels equivalent to those of the controls 1 h after the treatment (70.88 ± 5.02 in controls versus 68.49 ± 2.00 μg/g; nonsignificant).

Fig. 1

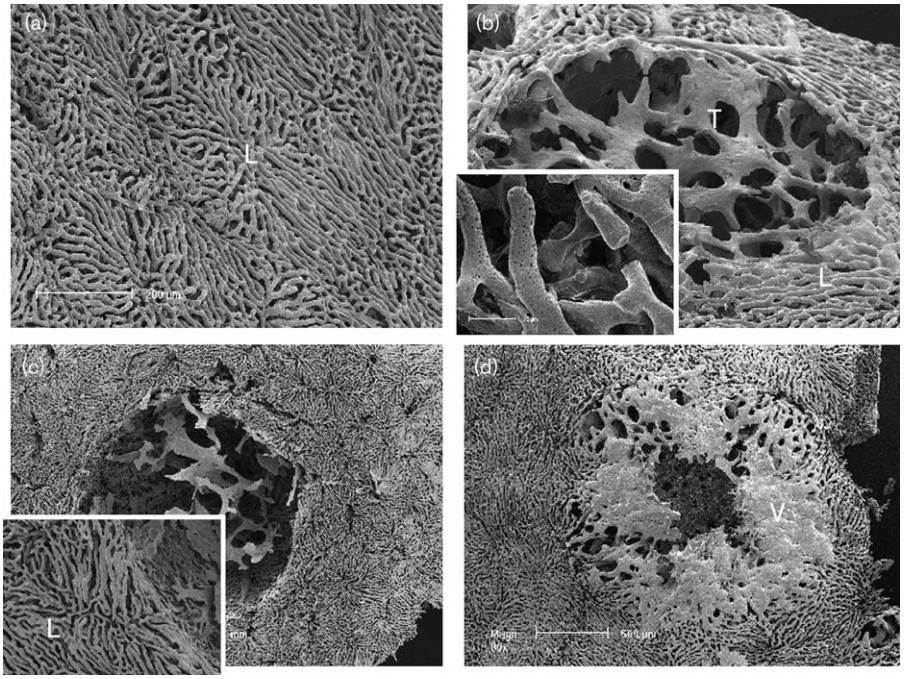


The effect of OXi4503 on tumor perfusion. Tumor-bearing mice were administered a single 100-mg/kg dose of OXi4503 and changes in blood flow were assessed 1 h and 5 days, respectively, after the treatment. Tumor perfusion was significantly reduced immediately after the treatment and was maintained 5 days later. $^{**}P < 0.001$.

Discussion

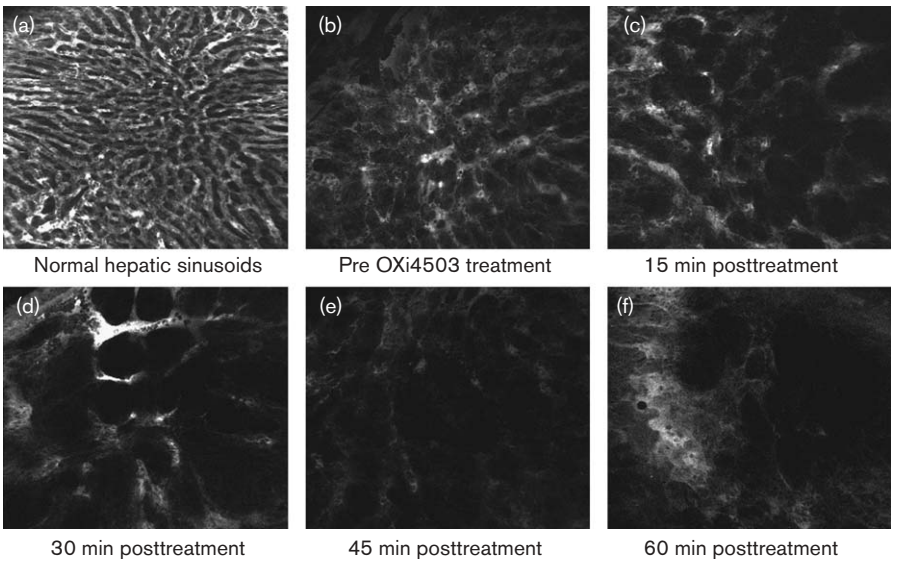
Tumors are dependent on their vascular supply for continual growth and metastatic potential. Selective targeting of mature tumor vessels is an alternative therapeutic approach to conventional chemotherapies in the treatment of solid tumors. VDAs are a family of compounds that have been shown to exert significant anticancer activity by specifically destroying the tumor blood vessels with subsequent necrosis [1].

Fig. 2



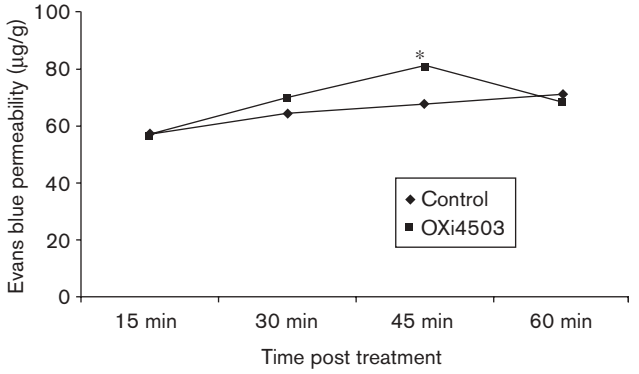
The effect of OXi4503 on tumor microvasculature. After administering a single 100-mg/kg dose of OXi4503, livers were perfused with an acrylic resin to form casts, which were subsequently viewed using a scanning electron microscope. Hepatic sinusoids show highly organized microvascular structure (a). This is in contrast to the spatially disorganized array of vessels in the tumor (b). Architectural damage was evident immediately after treatment with constricted vessels and filling defects indicative of tumor necrosis (c) No injury was evident in the normal liver area (c, *inset)*. Five days after treatment, revascularization had occurred, with the formation of large vascular lakes (d). L=normal liver, T=tumor vessels, N=necrosis, V=vascular lakes.

Fig. 3



The effect of OXi4503 on vascular permeability. In-vivo confocal microscopy was used to assess alterations in permeability after OXi4503 treatment at 15-min intervals. Unlike the normal hepatic sinusoids that show homogenous fluorescence intensity indicative of healthy, mature vessels (a), tumor vessels exhibit a heterogenous pattern of fluorescence with gradual reduction in intensity after OXi4503 treatment (b–f).

Fig. 4



The effect of OXi4503 on vascular permeability. The extent of vascular permeability after OXi4503 treatment was also evaluated by measuring the extravasation of dye using an Evans blue assay. A significant increase in permeability was only detected 45 min after OXi4503 treatment. *$P<0.05$.

The VDA OXi4503 belongs to a family known as combretastatins, which were originally derived from the bark of the South African Willow *Combretum caffrum* [18,19]. This agent has been shown to be highly selective for the tumor vessels, and causes immediate and extensive tumor vascular shutdown by binding to the internal tubulin cytoskeleton and inhibiting microtubule polymerization [20]. We have previously shown OXi4503 to retard tumor growth in a dose-dependent manner and to increase survival in a murine model of CRC liver metastases at a dose well below its MTD [10,16].

Despite causing marked tumor necrosis, the pattern of cell death is heterogenous and tumor regrowth occurs after the cessation of the OXi4503 treatment [7,13]. Immunolabeling for the vascular endothelium marker CD34 detected the presence of patent tumor vessels within the necrotic core [16], indicating that OXi4503 had not completely eradicated all the tumor vessels; this fact might have assisted in tumor regrowth.

In our study, a 30% reduction in perfusion was detected within 1 h of the OXi4503 treatment. A study by Sheng *et al.* [20] found a 50% reduction 1 h after treatment and an almost complete vascular shutdown within 24 h of using the same dose. The discrepancy can be attributed to the different tumor models used. Earlier studies have shown that subcutaneous and orthotopic tumors respond differently to OXi4503 treatment [7].

Despite the reduction in tumor blood flow being maintained for 5 days after the OXi4503 treatment, rapid tumor revascularization had taken place and large vascular lakes had formed. A recent study by Shaked *et al.* [21] found circulating progenitor endothelial cells migrating to the tumor rim after OXi4503 treatment and subsequently aiding the regeneration of the tumor vasculature. Although tumor revascularization had occurred, the blood flow was, nevertheless, still significantly reduced. This indicates that perfusion might get hindered or remain stagnant during the neovascularization process and that it only recovers once the vessel regeneration is complete.

An increase in permeability is a major characteristic in VDA therapy [22,23]. It can occur as a consequence of the morphological changes in the endothelial cells [24] or of the vascular shutdown-induced upregulation of the vascular endothelial growth factor [25]. OXi4503 markedly alters the tumor microvasculature and increases tumor necrosis within 1 h of treatment. Studies have, however, also shown marked increases in vascular permeability several hours after treatment [20]. Our results confirm that OXi4503 has extensive antivascular activity with minimal effect on normal vasculature. In our murine model of CRC liver metastases, significant alterations in blood flow and tumor vasculature were noted within 1 h of OXi4503 treatment. This was preceded by an increase in tumor permeability, which supports the observed elevation in necrosis observed 1 h after the OXi4503 treatment [16]. It seems as though the changes in vascular permeability are a major contributor to the underlying action of these agents. The ability of OXi4503 to induce vascular destruction along with an increase in their permeability might be beneficial for combination therapies.

The increase in permeability in tumor vasculature can enhance the penetration and accumulation of chemotherapeutic agents, especially of macromolecular anticancer agents such as liposomes and drug-conjugated high-molecular-weight polymers within the tumor [26]. The proceeding vascular shutdown can then impede the elimination of these agents from the tumor tissue and thereby prolong the therapeutic efficacy of the agent.

OXi4503 has been shown to cause substantial tumor destruction in many preclinical models; it is currently undergoing phase I clinical trials in patients with advanced cancer. In our study, we have found that even at the MTD of OXi4503 in our murine model of CRC liver metastases, incomplete vessel destruction was apparent. Also noted was the ability of OXi4503 to enhance vascular permeability. This supports further investigations into the synergistic effects of OXi4503 and other anticancer therapies such as chemotherapy.

Acknowledgements

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