

## Effect of Vascular Targeting Agent Oxi4503 on Tumor Cell Kinetics in a Mouse Model of Colorectal Liver Metastasis

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**Abstract.** *Background: Oxi4503 has been shown to inhibit tumor growth and improve survival in an animal model of colorectal (CRC) liver metastases. This agent appears to selectively target the endothelial cytoskeleton with resultant vessel occlusion and tumor necrosis. Materials and Methods: This study evaluated the pattern of tumor necrosis caused by Oxi4503, with particular emphasis on patterns of cell proliferation and apoptosis in a murine model of CRC liver metastases. Results: A single dose of Oxi4503 caused immediate tumor vasculature collapse and subsequently tumor necrosis. There was widespread central necrosis with evidence of viable tumor cells at the periphery. Alterations in the number and spatial pattern of tumor cells undergoing apoptosis and the rate of cellular proliferation were also observed following treatment. Microvessel density was reduced following treatment, however patent vessels were still observed within the necrotic core. Conclusion: Although Oxi4503 caused significant tumor destruction, synergistic treatment with cytotoxic and/or anti-angiogenic agents should be considered in order to achieve complete tumor eradication and long-term survival.*

The differential characteristic between the tumor vasculature and normal blood vessels has allowed the development of agents that can specifically target these abnormalities. Vascular targeting agents (VTA) are a large group of compounds that specifically target established tumor vasculature (1). These agents disrupt the tumor endothelium and occlude tumor blood flow, resulting in extensive ischemic necrosis of the tumor.

Oxi4503 is a VTA belonging to a family known as the combretastatins. Originally derived from the bark of the

South African Willow *Combretum caffrum* (2, 3), members from this family have been shown to be highly selective for tumor vasculature and cause significant tumor destruction with minimal side-effects (4-10). Oxi4503 selectively targets the endothelial cell cytoskeleton and causes morphological alterations which lead to tumor vessel occlusion and subsequent cellular necrosis (11). Despite causing significant tumor necrosis, the pattern of cell death is not homogenous and following cessation of Oxi4503 treatment, tumor regrowth occurs (9, 10, 12). The underlying mechanisms of cell death and patterns of necrosis remain to be elucidated.

Using a well established murine model of CRC liver metastases, the aim of this study was to determine the optimal dose of Oxi4503 causing growth retardation and tumor cell necrosis and to investigate the changes in the patterns of tumor cell death, apoptosis, proliferation and microvessel density following treatment with Oxi4503.

### Materials and Methods

**Animals.** Male inbred CBA mice (Laboratory Animal Services, University of Adelaide, South Australia) were maintained in standard cages with access to irradiated food and water *ad libitum*. All procedures performed were implemented in accordance with the guidelines of the Austin Hospital animal ethics committee.

**Experimental model of CRC liver metastasis.** The primary cell line (MoCR) was derived from a dimethyl hydrazine-induced primary colon carcinoma in the CBA mouse. Liver metastases were induced by a 0.05 mL intrasplenic injection of  $0.5 \times 10^6$  cell/mL cell suspension using a previously established technique (13). This model of liver metastasis closely resembles human disease and has been extensively characterized previously.

**Administration of Oxi4503.** Oxi4503 (Combretastatin A-1 trans-stilbene) kindly donated by Oxigene® Inc. was suspended in sterile saline and administered *via* intraperitoneal injections throughout the study.

**Study One: Determination of the single maximum-tolerated dose (MTD) of Oxi4503.** The tumor-induced mice were administered a single dose of Oxi4503 ranging from 50-200 mg/kg 16 days post-induction. The control groups were given an equivalent volume of

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**Key Words:** Oxi4503, vascular targeting agent, apoptosis, necrosis, colorectal liver metastasis.

Table I. Antibodies used for immunolabelling studies and assessment protocols.

	Antibody	Source	Incubation condition	Assessment method
Cellular proliferation	Ki-67 (Clone TEC-3)	Dako, Sydney, Australia	1:100, 3 hours	Percentage of positively nuclear stained cells in x200 magnification images.
Apoptosis	Active caspase 3 (AF835)	R & D Systems, Minneapolis, USA	1:1000, 30 minutes	Percentage of positively nuclear stained cells in x400 magnification images.
Microvessel density	CD34 (Clone MEC 14.7)	Dako, Sydney, Australia	1:500, 30 minutes	Number of positively stained patent vessels with visible lumen in x100 magnification images.

sterile saline. Volumetric assessments were made at the study end-point (21 days post-induction). A total of eight animals were used per treatment group.

*Study Two: The influence of Oxi4503 on tumor necrosis, apoptosis, cellular proliferation and vascular density.* The tumor-bearing mice were treated with the single MTD determined by study one, and assessed one hour or five days after treatment. Histology was used to quantify and examine the spatial pattern of tumor necrosis. Immunohistochemistry was used to ascertain the pattern of apoptosis, cellular proliferation and microvessel density.

*Volumetric assessment.* Quantitative stereological examination was conducted to determine the effect of Oxi4503 on several tumor parameters such as mean tumor volume, mean percentage of liver occupied with metastases and mean tumor size. Digital images of liver slices were assessed using an image analysis program (Image-Pro Plus Version 4.5, MediaCybernetics, CA, USA).

*Histological assessment.* Hematoxylin and eosin (H & E) stained sections were examined histologically and digital images captured using a Nikon Coolscope® (Nikon Corporation, Chiyokd-ku, Tokyo, Japan). The percentage of necrosis within each tumor was determined using an image analysis program (Image-Pro Plus). A minimum of 50 tumors were assessed per treatment group.

*Immunohistochemistry.* Formalin-fixed liver sections were embedded in paraffin and immunohistochemical staining was performed following the standard protocol. All incubations were performed at room temperature and negative controls consisted of omission of the primary antibodies. The antibodies used and assessment method are given in Table I. A minimum of ten tumors were assessed for each treatment group.

*Statistical analysis.* All data are represented as the mean±standard error of the mean. Statistical analysis was conducted using SPSS (Statistical Package for the Social Sciences™, version 10, Chicago, IL, USA) using both parametric and non-parametric analytical tests as appropriate. All statistical tests were two-sided and  $p<0.05$  was considered statistically significant.

## Results

*Determining the single MTD.* The LD<sub>50</sub> of Oxi4503 in this tumor model was estimated to be 200 mg/kg. The doses 50, 75 and 100 mg/kg were tolerated and showed a dose-dependent response with 100 mg/kg being the most effective at reducing tumor growth. Compared to controls, a single 100 mg/kg dose of Oxi4503 significantly reduced mean tumor volume ( $506.09 \text{ mm}^3 \pm 206.60$  versus  $1090.26 \text{ mm}^3 \pm 487.58$ ,  $p<0.001$ ) and percentage of liver occupied with metastases ( $15.50\% \pm 6.33$  versus  $18.87\% \pm 7.14$ ,  $p<0.05$ ) (Figure 1A and 1B). Tumor cross-sectional area was also markedly reduced ( $0.58 \text{ mm}^2 \pm 0.23$  versus  $1.72 \text{ mm}^2 \pm 0.77$ ,  $p<0.05$ ) (Figure 1C). It was concluded that 100 mg/kg Oxi4503 was the optimal single MTD. This dose was used as the basis for the remaining studies.

*Assessment of tumor necrosis and apoptosis following Oxi4503 treatment (acute and at D21).* There was a four-fold increase in the extent of tumor necrosis one hour after Oxi4503 treatment compared to controls ( $15.90\% \pm 1.06$  versus  $64.40\% \pm 2.29$ ,  $p<0.001$ ). However, the amount of necrosis had subsided by the study end-point ( $20.89\% \pm 1.98$  versus  $22.98\% \pm 2.12$ , non-significant) (Figure 2A).

In contrast to the untreated tumors (Figure 2B), characteristic central tumor necrosis was observed immediately following Oxi4503 treatment, with some tumors displaying complete cellular degradation and a small rim of viable tumor cells (Figure 2C and E). The extent of necrosis had diminished five days after treatment with clearance of the necrotic infiltrate and regrowth of tumor cells (Figure 2D). Despite the tumor recurrence, the overall tumor mass was considerably less dense than that of the untreated tumors with the development of granulation tissue in the Oxi4503-treated tumors (Figure 2D).

Immediately after Oxi4503 treatment, some hepatic sinusoidal damage was observed, especially in the

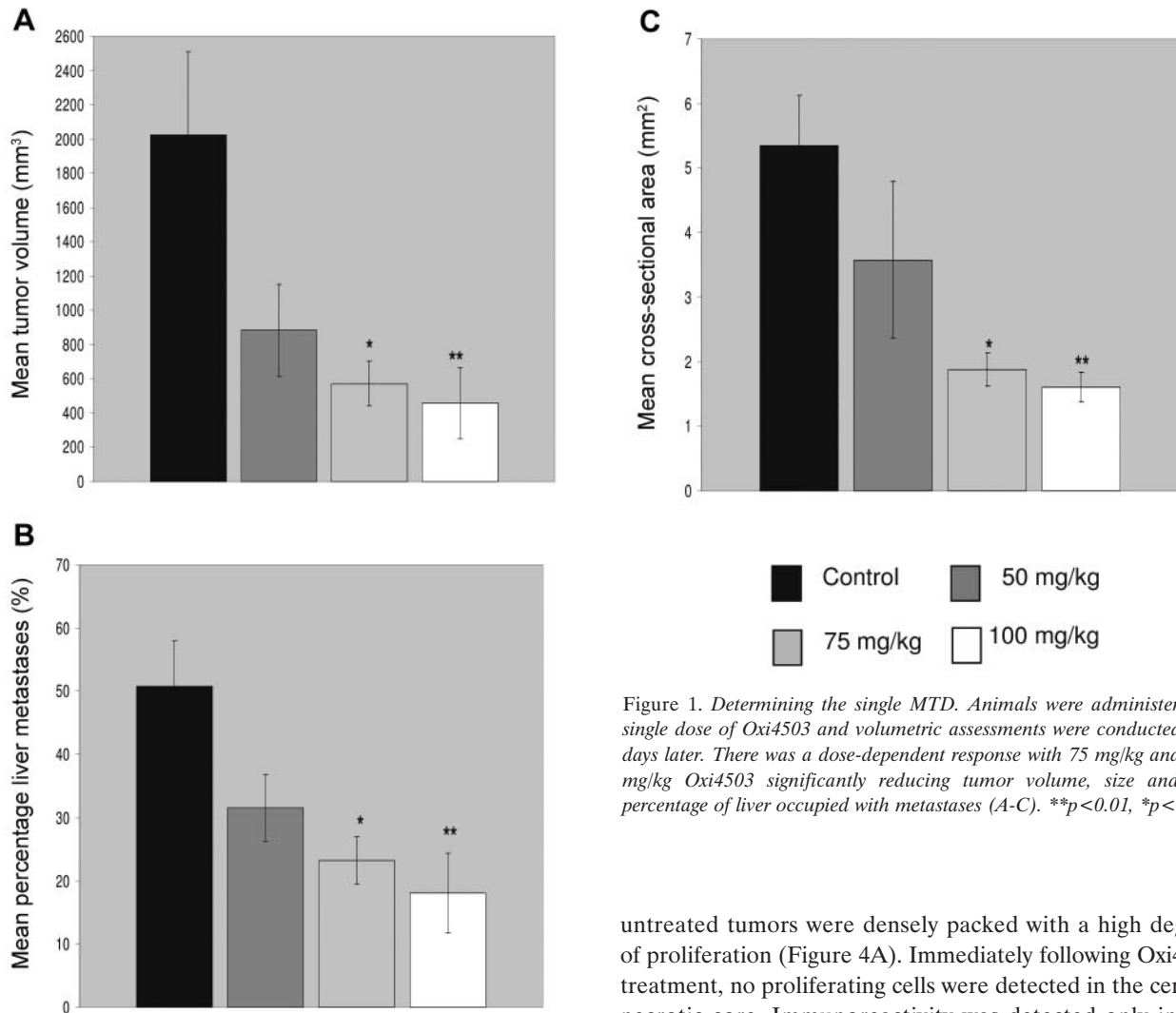


Figure 1. Determining the single MTD. Animals were administered a single dose of Oxi4503 and volumetric assessments were conducted five days later. There was a dose-dependent response with 75 mg/kg and 100 mg/kg Oxi4503 significantly reducing tumor volume, size and the percentage of liver occupied with metastases (A-C). \*\* $p < 0.01$ , \* $p < 0.05$

immediate areas surrounding the tumor (Figure 2E). This was, however, only evident in some tumors and was not apparent five days after treatment (Figure 2D).

Apoptosis was assessed by immunohistochemical staining for the apoptosis marker active caspase 3 and examination of cells using H & E for morphologically apoptotic cells (Figure 3A-D). In both assessments, apoptosis was found to be significantly elevated immediately following Oxi4503 treatment ( $0.74\% \pm 0.19$  in controls *versus*  $1.47\% \pm 0.35$ ,  $p < 0.05$ ) (Figure 3E and 3F). However, the elevated apoptotic rate had diminished to levels comparable to untreated samples five days post treatment ( $0.65\% \pm 0.13$  in controls *versus*  $1.09\% \pm 0.20$ , non-significant).

*Expression of cellular proliferation following Oxi4503 treatment (acute and at D21).* Ki-67 expression was examined at the same time-points as for cell death. Histologically, the

untreated tumors were densely packed with a high degree of proliferation (Figure 4A). Immediately following Oxi4503 treatment, no proliferating cells were detected in the central necrotic core. Immunoreactivity was detected only in the tumor rim (Figure 4B). By day 21, the degree of proliferation had increased with the distribution of cells exhibiting decreased density comparable to that of controls (Figure 4D and 4C, respectively).

Quantification of the percentage of proliferating tumor cells showed a significant decrease immediately after Oxi4503 treatment compared with the controls ( $43.57\% \pm 5.69$  *versus*  $61.52\% \pm 3.12$ ,  $p < 0.05$ ). Five days after treatment, although histologically the tumors showed reduced density, there was no significant difference between the tumors treated with Oxi4503 and the controls ( $58.08\% \pm 4.26$  *versus*  $65.50\% \pm 2.98$ , non-significant) (Figure 4E).

*Characterization of microvascular density following Oxi4503 treatment.* The tumors in this animal model of CRC liver metastasis were well-established and highly-vascularised beginning from day 16 (Figure 5B). After a single MTD of Oxi4503, despite substantial vascular occlusion and central necrosis, patent vessels were still detected within the

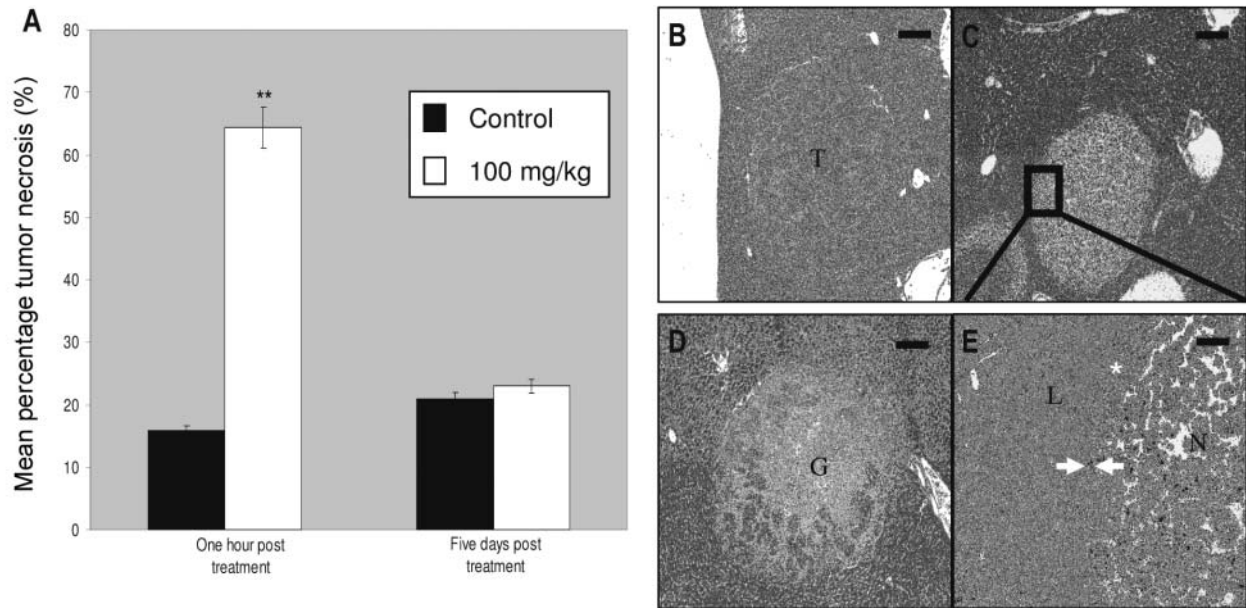


Figure 2. Effect of Oxi4503 on tumor necrosis. A representative sample of H & E stained sections was used to assess the percentage of tumor necrosis in controls and Oxi4503-treated groups. An increase in necrosis was observed immediately following treatment (A) in contrast to untreated samples (B) central tumor necrosis with a small rim of viable tumor cells was evident (C & E). Five days following treatment, the amount of necrosis had subsided with regrowth of residual tumor cells (D). Some normal liver damage was observed (E) which was not evident five days post treatment. T: tumor, L: liver, D: liver damage, \* viable rim, G: granulation tissue, arrows: tumor-host interface; bars (B-D) = 200 mm, (E) = 50 mm; \*\* $p < 0.01$ .

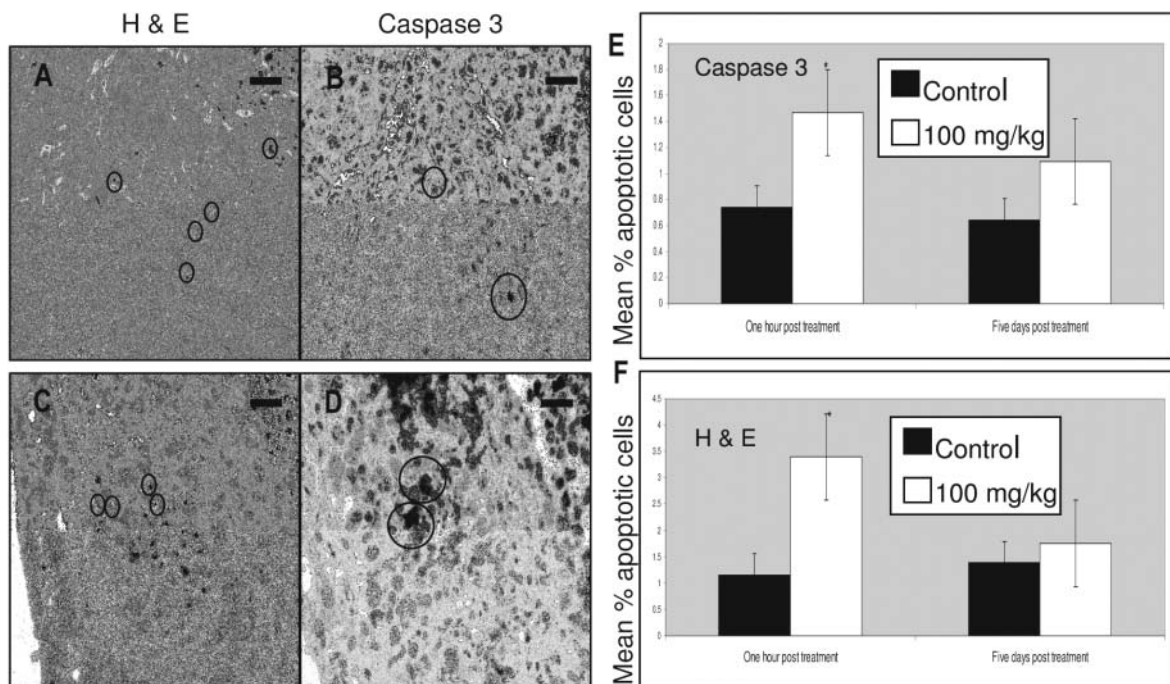


Figure 3. Effect of Oxi4503 on apoptosis. Immunolabelling using apoptotic marker active caspase 3 and assessment of H & E stained sections for morphologically apoptotic cells were used to examine extent of apoptosis following Oxi4503 treatment. Aberrant expression of apoptotic bodies were observed with no regular pattern (A-D). A marked increase in apoptosis was noted immediately after treatment which returned to pre-treatment levels five days after Oxi4503 treatment (E-F). Circle, apoptotic cells. Bars (A-D) = 25 mm. \* $p < 0.05$



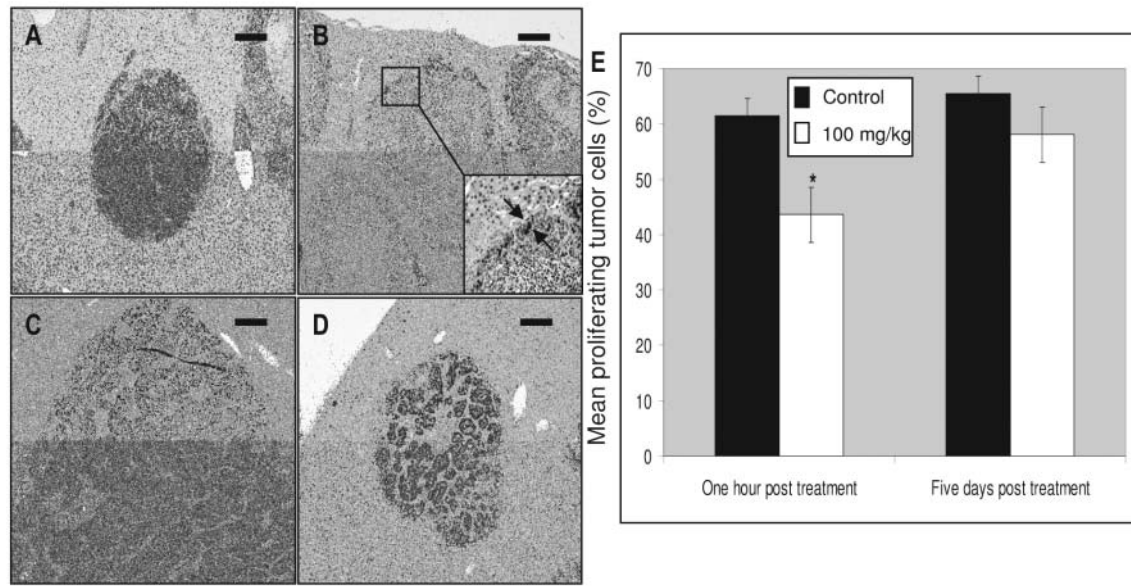


Figure 4. Effect of Oxi4503 on cellular proliferation. Following immunohistochemical staining using Ki-67, the pattern of proliferation and the amount of positively stained nuclei was examined. Untreated tumors were highly proliferative (A). One hour following Oxi4503 treatment, the extent of proliferation was confined to the tumor periphery (B). Five days following treatment, the extent of proliferation in the treated tumors was comparable to the untreated ones (C-D). Quantification of the percentage of proliferating cells showed a reduction in proliferation one hour following Oxi4503 treatment, but this had recovered by the study end-point (E). Arrows, viable tumor rim; bars (A-D) = 200 mm; \* $p < 0.01$ .

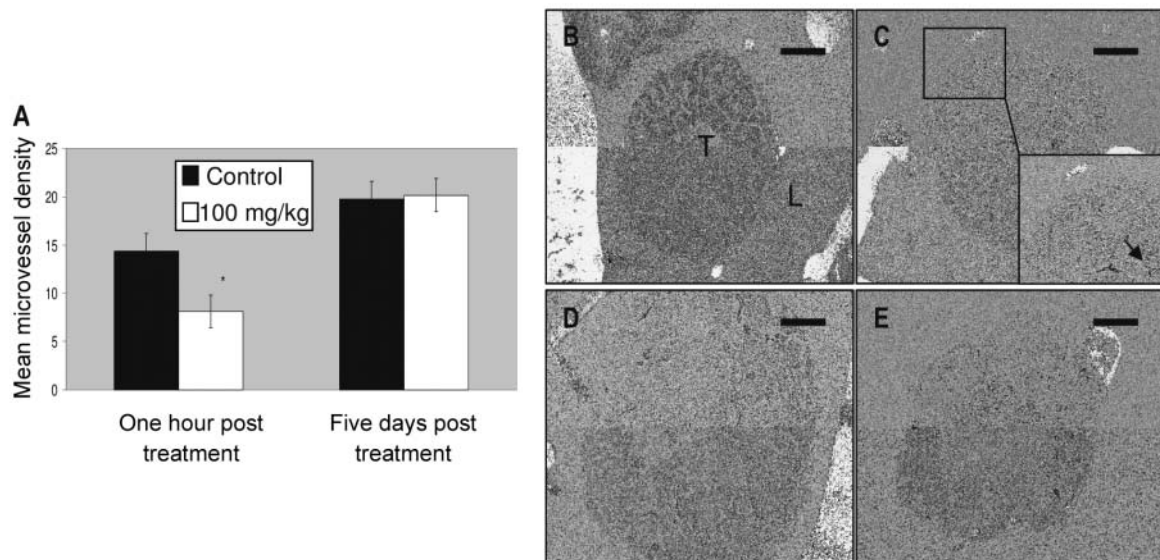


Figure 5. Effect of Oxi4503 on microvessel density. Microvessels were labeled using the vascular endothelium marker CD34. The microvessel density was examined by quantifying patent vessels, those with a visible lumen, within the tumor. Untreated samples at day 16 and 21 display highly vascularised tumors (B & D respectively). Despite the reduction in microvessels immediately following Oxi4503 treatment (A), patent vessels were still evident within the necrotic core (C). Five days after treatment, the extent of vascularisation was comparable to untreated tumors (E). Arrows, patent vessels; bars (B-E) = 200 mm; \* $p < 0.05$ .

central necrotic core (Figure 5C). By day 21, the degree of immunoreactivity had increased and was comparable to controls (Figure 5D and E). Quantification of the number of patent vessels found microvessel density was reduced

immediately following treatment ( $14.38 \pm 2.68$  in controls *versus*  $8.12 \pm 1.26$ ,  $p < 0.05$ ). However, by day 21, the microvessel density in the treated tumors was equivalent to controls ( $19.75 \pm 1.11$  *versus*  $20.16 \pm 2.20$ , non-significant).

## Discussion

Specific targeting of established tumor vessels is an alternative therapeutic approach to current strategies of chemotherapies. Unlike anti-angiogenic agents which prevent the formation of new tumor vessels (14, 15), VTAs selectively destroy the existing tumor vasculature by targeting the specific pathophysiological differences within the tumor endothelium (1).

Oxi4503 selectively destroys tumor vessels by binding to tubulin, causing microtubule depolymerisation and subsequently extensive tumor vascular occlusion at doses well below its MTD (3, 7, 9, 10). The vascular shutdown can be detected within minutes of Oxi4503 treatment and has been attributed to the immediate increase in tumor vascular permeability (11). The resulting imbalance between intravascular and interstitial fluid pressures, together with endothelial cell changes, can subsequently lead to ischemic necrosis and retardation in tumor growth. We have previously shown Oxi4503 to retard tumor growth significantly and increase survival in a murine model of CRC liver metastasis (16).

In this study, the single MTD of Oxi4503 was found to be 100 mg/kg which was consistent with a recent study by Shaked *et al.* (17) in contrast to a study by Hill *et al.* (7) in which Oxi4503 was found to be well tolerated up to a dose of 500 mg/kg. This disparity may be due to several factors. Firstly, during the induction of metastasis, the spleen is removed to prevent the formation of primary tumors. This absence may contribute to the reduced tolerance. In addition, our model was an orthotopic model and previous studies have shown Oxi4503 to exert different anti-tumor responses between subcutaneous and orthotopic tumors (10).

At the study end-point, Oxi4503-treated tumors were significantly smaller in size and volume than the untreated tumors being equivalent to the control samples at day 16. Despite being well tolerated, damage to the normal liver surrounding the tumor was observed immediately after Oxi4503 treatment, which was consistent with a study by Hua *et al.* (18). This indicates that despite being part of the normal vasculature, the peripheral vessels may also be susceptible to the anti-vascular effects of Oxi4503. It is also suggestive of Oxi4503 having cytotoxic activity despite being classed as an endothelium-specific agent (7). Due to the transient nature of Oxi4503 (11, 18), the injury to the normal liver was not apparent by day five following Oxi4503 treatment.

Although causing maximal necrosis extending to the tumor-host interface, the pattern of cell death was not homogenous, with a small population of tumor cells, mainly at the tumor periphery, surviving following cessation of Oxi4503 treatment, which is in agreement with other studies (7, 10, 18). The reduced susceptibility of these cells to Oxi4503 may be attributed to their close proximity to normal hepatic sinusoids. In the event of vascular occlusion

caused by Oxi4503, these cells have an alternate blood supply and are consequently less reliant on the tumor vasculature. These residual cells also rapidly replicate due to the release of angiogenic cytokines caused by the extensive vascular disruption (19, 20). The decrease in blood flow also enhances hypoxia which can promote the production of pro-angiogenic factors (20, 21). The incomplete tumor cell eradication following Oxi4503 treatment implies that spatial variations within the tumor microenvironment may confer an advantage to some tumor cells rendering them resistant to the effects of Oxi4503.

Previous studies have shown Oxi4503 to induce apoptosis in cultured human umbilical vein endothelial cells (10, 11). In our study, an increase in the number of apoptotic tumor cells was observed immediately following treatment. This may be consequential to the abrupt vascular disruption, which stresses the tumor cells and subsequently causes them to undergo apoptosis.

Despite Oxi4503 a VTA, complete vessel destruction was not observed and patent vessels were present within the central necrotic core. Variations in endothelial cell (EC) composition may render some cells resistant to the vascular disturbing effects of Oxi4503. Wehbe *et al.* (22) have demonstrated that alterations in the tubulin isotype within the EC can result in resistance to the structural analogue of Oxi4503, combretastatin A-4 phosphate treatment. Despite a reduction in patent vessels immediately following treatment, by day 21 there was no difference in the microvessel density in the treated group compared to the control samples. This is attributed to the occurrence of angiogenic regrowth following cessation of the treatment exacerbated by the up-regulation of growth factors within the tumor environment. In a recent study by Shaked *et al.* (17), four hours following Oxi4503 treatment, a significant increase in circulating endothelial progenitor (CEP) cells was observed. CEPs are bone marrow-derived cells that contribute to less than 5% of newly formed tumor vasculature (23). Following Oxi4503 treatment however, it was found that these cells not only migrated towards the viable rim, but were also a major contributor to the tumor regrowth by forming new tumor vasculature (17). This observation further supports the combining of Oxi4503 with other chemotherapeutic agents as an anti-cancer treatment modality since cytotoxic agents can target CEPs (24). However, careful timing would be essential to optimize the effects of treatment.

The combining of Oxi4503 with conventional cytotoxic therapy to target the residual population of tumor cells is a potential novel therapy modality. The synergistic use of two compounds that target different compartments of the tumor would not only maximize the anti-tumor activity, but might also reduce the dose of cytotoxic drugs needed for treatment. This would be beneficial for the patient as cytotoxic agents are commonly associated with severe side-

effects. A study by Shnyder *et al.* (25) found that Oxi4503 potentiated the anti-tumor properties of cisplatin. However, our study has demonstrated a significant reduction in the number of proliferating tumor cells immediately following Oxi4503 treatment which would hinder the effectiveness of cytotoxic therapy. Consequently, determination of the optimal time interval before administration of cytotoxic therapy following Oxi4503 treatment would be essential.

Our results have shown that a single dose of Oxi4503 can cause immediate alterations in tumor cell kinetics. Despite the substantial eradication of tumor cells, tumor regrowth following cessation of treatment is still evident. This remains a major challenge associated with VTA therapy and further investigations into accelerated tumor regrowth after Oxi4503 treatment are warranted.

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