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Evaluations of vascular disrupting agents CA4P and OXi4503 in renal cell carcinoma (Caki-1) using a silicon based microvascular casting technique

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ABSTRACT

The present study evaluated the treatment efficacy of the vascular disrupting agents CA4P and OXi4503 in an orthotopically transplanted human renal cell carcinoma xenograft model (Caki-1). Experiments used vascular casting, vessel density assessments as well as tumour necrosis measurements to evaluate the efficacy of these agents. After treatment with either agent, assessment of the vascular casts showed an almost total eradication of tumour blood vessels. Histological evidence further supported this observation, showing extensive central tumour necrosis with only a small viable rim of tumour cells remaining at the periphery. These results suggest that vascular disrupting agents CA4P and OXi4503 may have utility in the treatment of renal cell carcinoma, an encouraging result given that current conventional therapies have been currently largely unsuccessful in managing this disease.

1. Introduction

Renal cell carcinoma (RCC) is the most common malignancy of the kidney in adults and accounts for about 3% of all adult malignancies.[1] Unless detected at an early stage, at a time when it is a respectable neoplasm, RCC has a very unfavorable treatment outcome to conventional measures.[2] Unfortunately, RCC is characterised by a lack of early warning signs, resulting in a high proportion of patients already presenting with metastases at initial diagnosis.[2] As a consequence, RCC remains fatal in nearly 80% of its patients.[2–4] Recent histopathological studies of RCC have revealed it to be a highly vascularised neoplasm, with abundant angiogenesis and abnormal blood vessel development.[5,6] One possible approach to improve treatment outcome might be the implementation of vascular targeting strategies.[7,8]

Vascular targeting therapies specifically target and exploit tumour vasculature abnormalities,[8–10] either though the inhibition of the development of new tumour vasculature (anti-angiogenic agents)[11] or through the destruction of the existing tumour vasculature (vascular disrupting agents).[12] One class of vascular disrupting agents (VDAs) and the focus of the present study are the small molecule tubulin binding agents. These agents act by disorganising the microtubules within endothelial cells; specifically they bind to the β-tubulin sub-units, preventing the formation of microtubules.[13–15]

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After treatment with such agents, an increase in vascular permeability is observed.[14] This observation is thought to be the result of newly formed daughter endothelial cells rounding-up as a consequence of cytoskeletal alterations.[14–16] The rounding of the endothelial cells results in an increased endothelial cell separation, followed by their detachment from the vascular wall. This detachment leads to a collapse of the vascular wall and results in obstruction of tumour blood flow.[7,10] Ultimately, the reduction in blood flow leads to tumour cell death through oxygen and nutrient deprivation as well as an obstruction for metabolic waste egress. Previous studies have reported that tumour cell death occurs within hours of agent delivery, resulting in extensive tumour necrosis by 24 h post treatment.[7]

A number of agents exhibiting these characteristics have been identified and after extensive preclinical testing, lead agents have begun to enter clinical trials.[17,18] Two promising agents under investigation are CA4P[7,10,19] and OXi4503.[20–22] The present study examined the treatment efficacy of these agents in a human RCC xenograft model (Caki-1).

2. Materials and Methods

2.1. Animal and tumour models

Female nude mice (NCR, nu/nu), age 6–8 weeks were maintained under specific-pathogen-free conditions (University of Florida Health Science Center) with food and water supplied *ad libitum*. Animals were inoculated with 2×10^6 tumour cells (suspended in 20 μl 0.9% saline) either orthotopically in the left kidney (injected through the skin) or intra muscularly (IM) in the left hind limb. Once the tumours reached a size ~200 mm^3, experiments were initiated. All research was governed by the principles of the Guide for the Care and Use of Laboratory Animals and approved by the University of Florida Institutional Animal Care and Use Committee (IACUC).

2.2. Treatment protocol

Tumour-bearing mice were randomly allocated to various experimental groups. Both CA4P and OXi4503 (OXiGENE, Waltham, MA 02451, United States of America) were prepared in saline and administered by intra-peritoneal injection (0.01 ml/g body weight) at doses of 100 mg/kg and 25 mg/kg, respectively. For all experiments, tumours were assessed 24 h after treatment; the time of maximum tumour necrosis.[7,23]

2.3. Tumour growth experiment

Preliminary tumour growth experiments were carried out to ascertain the time taken for orthotopically grown Caki-1 tumour to grow to ~200 mm^3. Mice were killed at various times after tumour cell inoculations, after which time their kidneys were removed and their respective tumour volumes assessed. Tumour volume was determined by fully submersing either the normal or tumour-bearing kidney in a measuring cylinder containing PBS. The tumour volume was then recorded as the difference between the tumour-bearing and normal kidney's fluid displacement volume.

Tumours initiated in the hind legs were measured every day by passing the tumour-bearing leg through a series of increasing diameter holes in a plastic plate. The smallest diameter hole the tumour-bearing leg could pass through was recorded and converted to a tumour volume using the formula: tumour volume = $(1/6)\pi d^3 - 100$, were d is the hole diameter and 100 represents a volume correction factor determined for mouse leg without a tumour.

2.4. Clonogenic cell survival

Tumour-bearing mice were killed 24 h after treatment. The tumours were removed and dissociated with an enzyme cocktail consisting of 0.025% collagenase (Sigma), 0.05% pronase (Sigma), and 0.04% DNase (Sigma). The cells were then counted and various dilutions were plated in 60 mm dishes. After 2 weeks of incubation at 37 °C, colonies of 50 or more cells were counted and cell survival was calculated. Tumour surviving fraction was corrected for tumour cell recovery.

2.5. Vascular casting, imaging and quantification

The procedure for casting and imaging as well as the quantification of the vascular casts, have been previously described in detail.[24] A brief summary of the methods used is given.

2.5.1. Vascular casting

Mice were anesthetised using an intra-peritoneal injection of ketamine (95 mg/kg) plus xylazine (5 mg/kg). Anaesthesia was maintained by inhalation of 1% isoflurane and oxygen administered *via* facemask. Mice were then infused in the left ventricle of the heart with 3 ml of the casting agent Microfil® (MV-120, Flow Tech, Inc., P.O. Box 834 Carver, MA 02330-0834, USA). After infusion, the cadavers were refrigerated for a 24 h period before kidney removal to allow time for the cast compound to fully harden.

2.5.2. Vessel imaging

Frozen sections of the microvascular casts were cut at 100 μm and mounted on 'Plus' slides (Fisher Scientific). The sections were first dried in a series of graded alcohol washes, before immersing them in methyl salicylate (Sigma) for a 24 h period. The sections were then imaged at 5× magnification using a morphometric 'tiled field mapping' microscope (Brain Institute, University of Florida). Each section was imaged by taking a weighted average from a series of 5 μm slices captured from top to bottom through the tissue sample. To aid quantification, each of the output images was fitted with a calibration scale.

2.5.3. Vessel size quantification

Images of the microvascular casts were measured using the chord-length distribution analysis incorporating the trilinear interpolation marching-cube (TRI-MC) smoothing algorithm.[25] Briefly, chord-lengths distributions were defined by the intersection of imaginary computer generated straight lines within the object's boundaries. The distance along each individual chord-length as it passes from one object boundary to the next was recorded. By repeating this process millions of times, a distribution of vessel sizes was generated.

2.5.4. *Vascular density measurements*

Vascular density measurements were made on the vascular casts using the Chalkley counting technique, where vascular density was recorded as the number of vessels per unit area.[24] For each tumour, the mean vessel density was determined based on a minimum of 10 random fields per section and 3 sections per tumour.

2.5.5. *Viable rim assessment*

Tumours were removed and fixed in 10% alcohol formalin for 24 h before sectioning. Paraffin sections were taken from the center of each tumour, stained using hematoxylin and eosin (H&E) and imaged on a morphometric microscope at 5× magnification using a tile field mapping technique.

2.5.6. *Statistical analysis*

The results were analysed using ANOVA in combination with Scheffe's post hoc procedure, and expressed as mean ± standard deviation. Differences were considered statistically significant at $P < 0.05$.

3. Results

Initial experiments established the impact of VDA treatment on tumour cell kill in IM or orthotopically grown Caki-1 tumours using the clonogenic cell survival assay. The response to CA4P (100 mg/kg) and OXi4503 (25 mg/kg) was assessed 24 h after drug exposure. These doses were chosen since they were shown to result in similar levels of tumour cell kill irrespective of the site of tumour growth (Fig. 1).

In subsequent studies the vascular casting technique was applied to visualise the vasculature in both normal kidney and renal cell carcinoma growing in the kidney before and after VDA treatment. Fig. 2a and e shows the vascular cast and histology of the cortex of a normal mouse kidney. Closer examination of the vascular cast shows that the casting medium employed was able to penetrate into the smallest of capillaries and reveal the internal structures of the individual glomeruli. The growth of RCC Caki-1 in the kidney is illustrated in Fig. 2b. The impact of treatment with either CA4P (100 mg/kg) or OXi4503 (25 mg/kg) on tumour vasculature is illustrated in Fig. 2c and d. The region of vascular destruction with its almost total elimination is readily apparent (Fig. 2c and d). The corresponding histology (Fig. 2g and h) is consistent with these observations revealing only a small viable rim of tumour tissue at the periphery after treatment with either agent.

To quantify the casting images, the chord-length distribution technique was used.[24] This analysis examined areas of normal kidney cortex, kidney cortex containing an untreated Caki-1 malignancy, and kidney cortex containing treated Caki-1 malignancies. Fig. 3 shows the results of this chord-length distribution analysis. The tumours from untreated

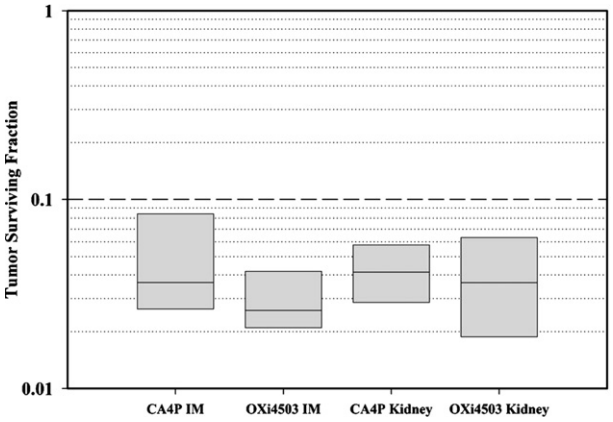

Fig. 1 – Clonogenic cell survival of Caki-1 tumour cells grown IM or orthotopically in the kidney 24 h following treatment with CA4P or OXi4503. Data represent the median of 4–5 tumours (line), 25–75% (box).

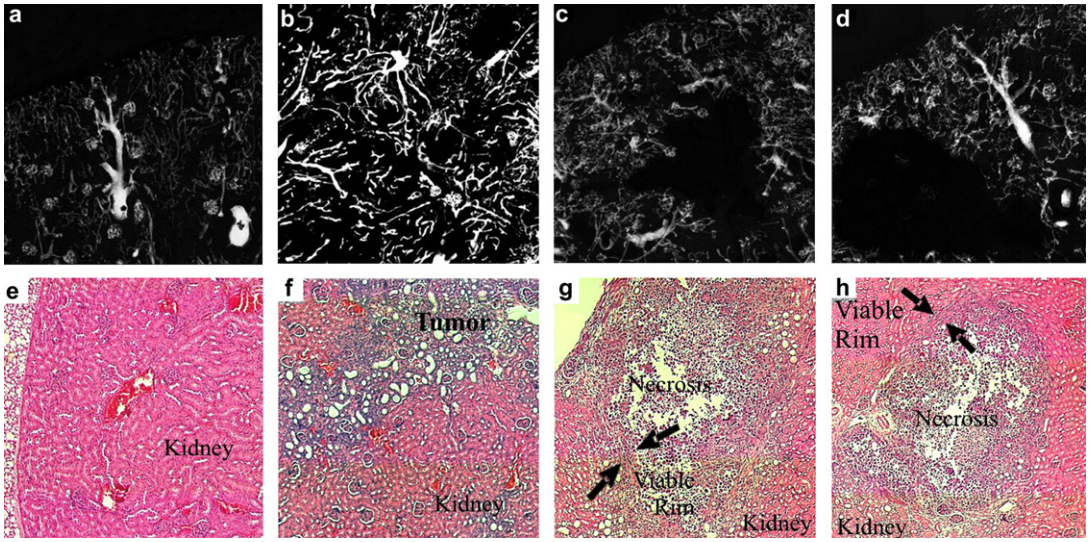

Fig. 2 – Images of silicon based vascular casts and histology: (a, b) normal kidney cortex; (c, d) Caki-1 growing in the cortex of the mouse kidney; (e, f) Caki-1 growing in the cortex of the mouse kidney 24 h after treatment with CA4P (100 mg/kg); (g, h) Caki-1 growing in the cortex of the mouse kidney 24 h after treatment with OXi4503 (25 mg/kg).

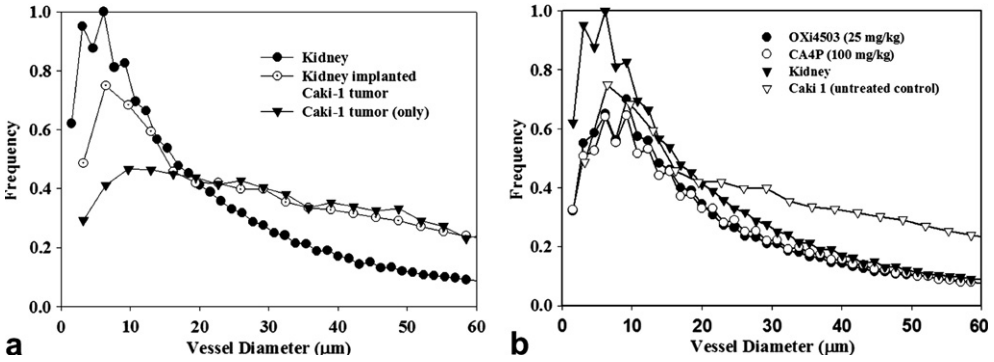

Fig. 3 – Vessel diameter distribution curves in normal kidney cortex, kidney implanted Caki-1 tumours and Caki-1 tumours in untreated mice (a) or in animals 24 h after treatment with either CA4P (100 mg/kg) or OXi4503 (25 mg/kg) (b).

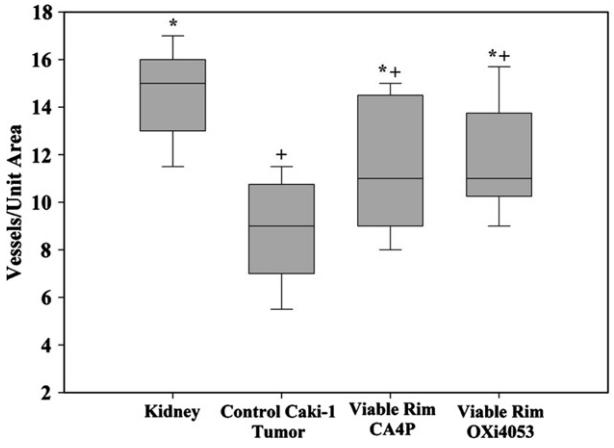

Fig. 4 – Vascular densities determined by Chalkley counting for both normal kidney cortex, untreated Caki-1 tumours and at the periphery of the tumours 24 h after treatment with either CA4P (100 mg/kg) or OXi4503 (25 mg/kg). Data represent the mean of 3–6 tumours (line), 25–75% (box) and 10–90 % (error bars); * statistically significant from normal kidney ($P < 0.05$), + statistically significant from control tumour ($P < 0.05$).

mice show a significant increase in the number of larger patent tumour blood vessels over that seen in the normal kidney tissue (Fig. 3a). In contrast, the profiles from tumours derived from mice treated with either CA4P (100 mg/kg) or OXi4503 (25 mg/kg) are very similar, indeed almost identical to those obtained from normal cortex without a tumour (Fig. 3b).

Finally, images of the vascular casts were used to determine the vascular density at the tumour periphery after treatment with either agent. These data were compared with our previous published results[24] which examined the vascular density within normal kidney cortex and untreated Caki-1 tumours grown within the cortex of nude mice. The results showed that while the vascular density within this viable rim is less than that of the normal tissue, it was still significantly greater than that found in the tumours of untreated mice (Fig. 4).

4. Discussion

The unsatisfactory management of renal cell carcinoma (RCC) with conventional anti-cancer therapies warrants the use of novel approaches to augment treatment response.[26] Previous studies have shown that RCC is a highly vascularised neoplasm containing vessel abnormalities common to many solid tumours.[6,24] Hence, vascular targeting therapies have been considered for treating this malignancy. The current study examined the use of two tubulin binding VDAs in a human renal cell carcinoma model grown orthotopically in nude mice.

VDAs like CA4P have been studied extensively in a variety of pre-clinical settings.[7,8] Their action is directed at the actively dividing endothelial cells, a cell population which is known to be considerably higher in tumours than normal tissue.[27] VDAs damage the established vasculature of solid tumours resulting in blood flow shut-down and subsequent tumour cell death due to induced ischemia. Initial experiments studied this effect in Caki-1 tumours grown either orthotopically or through IM (Fig. 1). Doses of 100 mg/kg (CA4P) and 25 mg/kg (OXi4503) were chosen, respectively, because previous studies had indicated that such doses resulted in maximal inductions of tumour necrosis and similar anti-tumour effects.[23,28] The relative difference in potency of the two agents has been attributed to OXi4503 being metabolised to a more reactive species than CA4P, though the exact mechanisms involved remain to be elucidated.[29] The results of the present studies showed that the efficacy of these agents, as determined by clonogenic cell survival, was independent of the site of tumour implantation. This result is not surprising since both agents are specifically targeting the tumour vascular and therefore should not be dependent on the site of tumour growth.

To evaluate the effects of these agents on tumour vasculature, a vascular casting technique was used. Mice were treated with CA4P (100 mg/kg) and OXi4503 (25 mg/kg) and vessel casts were made 24 h later (Fig. 2a–d). In parallel, these anti-vascular effects were also examined by means of histology (Fig. 2e–h). The results obtained with both these end-points were complimentary; i.e. together they clearly illustrated the loss of tumour vasculature and induction of wide scale necrosis in the central regions of the tumour, with

only a small viable rim of tumour cells remaining at the tumour periphery.

Examination of the tumour necrosis data (Fig. 2g–h) clearly shows that after treatment with these agents, a rim of viable tumour cells remains at the periphery of the tumour. It has been suggested that the viable rim arises because cells located at the periphery of the tumour acquire their nutritional support from blood vessels in the surrounding normal tissue.[23] However, the results of the vascular density studies indicated a lower vascular density at the tumour periphery after treatment with either agent than compared to the surrounding normal tissue. This observation may be the results of invading tumour cells increasing the inter vascular spacing between normal blood vessels located within this region. If true this would suggest that the viable rim of tumour cells seen after treatment may contain some normal blood vessels.

The effect of VDA treatment on the tumour blood vessel network was further evaluated using the previously described 'chord-length distribution technique'.[24] Profiles of vessel size with respect to their frequency of occurrence were generated for each of the experimental groups. Images of the vascular cast taken after treatment appear to show only traces of tumour vasculature at the periphery of the tumour. For this reason, the region of interest used for these investigations included some of the surrounding normal kidney vasculature. As a result the two tumour vessel profiles shown in Fig. 3a differ in that the one containing the surrounding normal kidney vasculature has a high number of small vessels. However, at larger vessel sizes the tumour vasculature begins to dominate and consequently there is little difference between the two curves (Fig. 3a). After treatment with the VDAs (Fig. 3b) the shapes of the distribution curves (at greater than 15 μm) were closely aligned with the profile of vessels obtained in scans of the normal kidney cortex. This result would suggest that the larger, tumour-associated blood vessels were eradicated, a theory that is supported by Fig. 2c and d, which show the destruction of the tumour vascular after treatment. Although it has been noted that tumour vasculature can be re-established from the surviving viable rim of tumour tissue,[7] recent studies in the KHT sarcoma model have shown that the newly formed tumour blood vessel network remains susceptible to subsequent VDA treatment.[28]

In conclusion, the results of the vascular casting and necrosis data presented in this study have demonstrated the efficacy of CA4P and OXi4503 treatment in a model of RCC, a promising result given the current failure to manage this malignancy in the clinic by conventional means. Both agents appear effective at inducing responses in the Caki-1 model, though OXi4503 does so at ~4-fold lower doses. Whether this will translate into a greater therapeutic gain will not be resolved until comparable normal tissue toxicity assessments are made. Still, the remnants of viable tumour tissue surviving either VDA likely means that both agents will have their greatest utility in the clinic when combined with conventional anti-cancer treatment modalities.

Conflict of interest statement

For all authors 'none declared'.

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