Vascular Disruption in Combination with mTOR Inhibition in Renal Cell Carcinoma

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ABSTRACT

Renal cell carcinoma (RCC) is an angiogenesis-dependent and hypoxia-driven malignancy. As a result, there has been an increased interest in the use of antiangiogenic agents for the management of RCC in patients. However, the activity of tumor-vascular disrupting agents (tumor-VDAs) has not been extensively examined against RCC. In this study, we investigated the therapeutic efficacy of the tumor-VDA ASA404 (DMXAA, 5,6-dimethylxanthenone-4-acetic acid, or Vadimezan) in combination with the mTOR inhibitor Everolimus (RAD001) against RCC. In vitro studies were carried out using human umbilical vein endothelial cells (HUVECs) and in vivo studies using orthotopic RENCA tumors and IH patient tumor-derived RCC xenografts. Magnetic resonance imaging (MRI) was used to characterize the vascular response of orthotopic RENCA xenografts to combination treatment. Therapeutic efficacy was determined by tumor growth measurements and histopathologic evaluation. ASA404/everolimus combination resulted in enhanced inhibition of endothelial cell sprouting in the 3-dimensional (3-D) spheroid assay. MRI of orthotopic RENCA xenografts revealed an early increase in permeability 4 hours post treatment with ASA404, but not with everolimus. Twenty four hours after treatment, a significant reduction in blood volume was observed with combination treatment. Correlative CD31/NG2 staining of tumor sections confirmed marked vascular damage following combination therapy. Histologic sections showed extensive necrosis and a reduction in the viable rim following combination treatment compared to VDA treatment alone. These results demonstrate the potential of combining tumor-VDAs with mTOR inhibitors in RCC. Further investigation into this novel combination strategy is warranted.
INTRODUCTION

Renal cell carcinoma (RCC) is a lethal genitourinary malignancy that accounts for 85% of primary renal neoplasms (1). In 2010, 58,240 patients were diagnosed with RCC in the US, with 13,040 reported cancer-related deaths (2). Improved understanding of the biology of RCCs over the last decade has resulted in increased investigation into the use of targeted therapeutics for RCC. Primary and metastatic RCCs are angiogenesis-dependent and hypoxia-driven malignancies (3,4). As a result, several agents targeting tumor angiogenesis are being actively investigated against RCC in preclinical and clinical studies (4, 5). These include inhibitors of the mammalian target of rapamycin (mTOR) and vascular endothelial growth factor (VEGF) pathways. Targeted agents that have recently received approval by the Food and Drug Administration (FDA) for clinical use include the mTOR inhibitors, everolimus and temsirolimus and the receptor tyrosine kinase inhibitors (TKIs), sorafenib and sunitinib. While TKI and mTOR inhibitors have demonstrated clinical benefit in RCC, patients with advanced disease fail these targeted therapies and develop refractory disease. Therefore, investigation into novel combination treatment approaches to improve clinical outcome remains a high priority.

The significance of the mTOR signaling pathway in RCC is well recognized (6). Pharmacologic inhibition of mTOR has been shown to inhibit tumor growth in preclinical models and in patients with RCC (6-8). In addition to their direct antitumor effects, mTOR inhibitors have been shown to exhibit also significant antiangiogenic activity (9, 10). Rapamycin and its analogues have a direct inhibitory effect of endothelial cell proliferation. mTOR pathway inhibition also impairs protein synthesis of angiogenesis related factors including HIF (11).
Tumor vascular disrupting agents (tumor-VDAs) constitute a unique class of drugs that are being considered as a valid therapeutic strategy for clinical development (12). Tumor-VDAs target established tumor vasculature, which is required by the tumor to survive and grow (12, 13). This action is distinct from that of anti-angiogenic agents, which predominantly target neo-vascularization (12-14). One such tumor-VDA is the small molecule ASA404 (vadimezan, 5,6-dimethylxanthenone-4-acetic acid; DMXAA), that has been shown to exhibit potent antitumor activity in combination with chemotherapy in preclinical models (15, 16). ASA404 induces apoptosis of tumor vascular endothelial cells, cytokine production and tumor vascular collapse – effects that culminate in tumor necrosis in vivo (17-19).

Since mTOR inhibitors and tumor-VDAs target distinct tumor vascular networks, we investigated the therapeutic potential of ASA404 in combination with the mTOR inhibitor, everolimus (RAD001). It was our hypothesis that combined destruction of established vessels and inhibition of angiogenesis would result in improved antitumor activity against RCC. To test this hypothesis, studies were carried out using endothelial cells in vitro and in vivo using two different RCC model systems. Experimental studies were carried out in vitro using human umbilical vein endothelial cells (HUVECs) and in vivo using orthotopic RENCA tumors and IH patient tumor-derived RCC xenografts. Magnetic resonance imaging (MRI) was used to characterize the vascular response of orthotopic RENCA xenografts to combination treatment. Therapeutic efficacy was determined by tumor growth measurements and histopathologic evaluation.
MATERIALS AND METHODS

Cell Culture and Reagents. The murine renal cell carcinoma (RENCA) cell line was purchased from ATCC and maintained in RPMI culture media (Life Technologies) supplemented with 10% fetal bovine serum, 1% pen/strep and incubated at 37°C in 5% CO₂. HUVECs were obtained from Lonza and maintained in EBM2 media (Lonza) with all included growth factors. No further authentication was performed on these cell lines. ASA404 (Vadimezan, formerly known as DMXAA,) and everolimus were kindly provided by Novartis Institute of Biomedical Research, Basel, Switzerland. The MR contrast agent, albumin-(GdDTPA)₃₅ was provided by the Contrast Media Laboratory, UCSF, San Francisco, CA (Drs. Robert Brasch and Yanjun Fu). For in vitro work, everolimus stock was made in DMSO and aliquoted and stored at -20°C. ASA404 was made fresh on the day of treatment in culture medium. For in vivo experiments ASA404 was administered weekly at 22 mg/kg or 25 mg/kg in 5% sodium bicarbonate solution (from 7.5% stock solution w/v, Invitrogen) by intraperitoneal injection. Everolimus was freshly prepared and administered in vivo by oral gavage in distilled water at 2.5 mg/kg on a 5 days on/2 days off schedule.

Three dimensional (3-D) spheroid assay. Tissue culture flasks were coated with 0.5% gelatin in PBS and HUVECs were propagated in EBM2 containing all the growth factors. Hydrated Cytodextrin beads were equilibrated with complete EBM2 medium without VEGF and were incubated with HUVECs for 4 hours in a 37°C incubator. Spheroids comprising the beads and HUVECs were embedded in fibrin after activation of fibrinogen in the presence of thrombin in 48 well plates. Endothelial cells were allowed
to sprout and form lumen when treatment was started alone or in combination. ASA404 and everolimus were dissolved in EBM2. Following treatment, plates were fixed in formalin and stained with DAPI in PBS. Pictures were captured on a Nikon E 2000 microscope and quantitation was performed using Image J software (NIH, Bethesda, Maryland).

**Immunoblotting:** Human umbilical vein endothelial cells (HUVEC) were treated with 100 µM ASA404, 1 nM everolimus or combination for 24 hours. HUVECs were washed in PBS and lysed in RIPA buffer (Sigma-Aldrich) containing 1x protease and phosphatase inhibitors (Sigma-Aldrich). Equal amounts of protein were separated by electrophoresis using 4-15% SDS-PAGE gradient gels (Bio-rad). Protein was transferred to nitrocellulose membranes and blocked with 5% nonfat milk in a Tris-buffered saline solution containing 0.1% Tween-20 for 2 hours. Immunoblotting was performed with anti-phospho-4EBP1 (Cell Signaling), anti-phospho-S6RP (Cell Signaling) and anti-β-actin (Sigma-Aldrich). Anti-rabbit and mouse horseradish peroxidase-conjugated secondary antibodies were from Dako (Carpinteria, CA). Immunoblots were visualized using enhanced chemiluminescence (PerkinElmer).

**In vivo animal models.** The Institute Animal Care and Use Committee (IACUC) at Roswell Park Cancer Institute (RPCI) approved all mouse protocols used in this study. Female 4 to 6 week old BALB/c mice (National Cancer Institute) were kept in a temperature-controlled room on a 12/12 h light/dark schedule with food and water *ad libitum*. Orthotopic implantation of RENCA cells has been previously described (20). Briefly, RENCA cells (5 x 10⁴) harvested from nonconfluent monolayer cell cultures in
50 µL of medium were injected under the renal capsule and tumor uptake and growth was monitored by palpation. The human xenograft model designated IH (21) was also utilized. Female 4 to 6 week old athymic nude mice were purchased from the National Cancer Institute (Frederick). IH tumor pieces 3 × 3 × 3 mm were transplanted into a new cohort of mice for expansion and eventual drug studies. Before starting the treatment within either model, tumor bearing mice were divided into homogenous groups (8–9 per group) according to tumor burden determined by size.

For in vivo studies, tumor bearing mice were treated with ASA404 (22 mg/kg once weekly; i.p. injection), everolimus (2.5 mg/kg 5d on 2d off; oral gavage) or combination for approximately 10d in the RENCA model and 35d in the IH model weeks. Mice were monitored for toxicity by weekly body weight measurements and tumor growth was measured twice weekly. Tumor tissue was harvested, weighed and fixed in 10% normal buffered formalin before being embedded in paraffin. Four (4 µm) samples were stained with hematoxylin and eosin for further analysis. Image acquisition was performed by using a Scanscope XT system (Aperio Imaging) and analyzed using Imagescope software (Aperio).

Bioluminescence imaging. Serial bioluminescence imaging was performed using the Xenogen IVIS® in vivo Imaging System (Caliper Life Science, Hopkinton, MA). Animals were injected IP with D-luciferin potassium salt dissolved in PBS. Ten minutes after D-luciferin injection, mice were imaged under isoflurane inhalational anesthesia for detection of luciferase activity (20).
**Magnetic resonance imaging (MRI).** MRI studies were performed using a 4.7-T/33-cm horizontal bore magnet (GE NMR Instruments, Fremont, CA, USA) incorporating AVANCE digital electronics (Bruker Biospec, ParaVision; Bruker Medical, Billerica, MA, USA). Induction of anesthesia prior to imaging and maintenance of anesthesia during imaging was achieved by inhalation of isoflurane (~2-3% in oxygen). Anesthetized animals were placed on an acrylic sled equipped with respiratory and temperature sensors and positioned within the magnet. An air heater system was used to maintain animal temperature in conjunction with the sensors embedded within the sled which provided continuous feedback during imaging.

Preliminary scout images were acquired on the sagittal and axial planes to assist in slice prescription for subsequent scans. Multislice non contrast-enhanced T2-weighted images were acquired on the coronal and the axial planes with the following parameters: TE/TR = 41/2500 ms, matrix size 256 x 192, 1 mm thick slices, FOV 3.2 x 3.2 cm, NEX = 4. T1-relaxation rates (R1) were measured using a saturation recovery, fast spin echo sequence before and after administration of the intravascular MR contrast agent, albumin-(GdDTPA)$_{35}$ (0.05 mmol/kg) as described previously (21). Image processing and analysis were carried out using commercially available software (AnalyzePC; AnalyzeDirect, OverlandPark, KS). Raw data were reformatted and object maps of desired regions of interest were outlined. Signal intensities from regions of interest were obtained and mean intensity within the regions of interest was used for calculating the T1 relaxation at each TR time. The change in relaxation rate (ΔR1) was then calculated for tumor and normalized to the contralateral kidneys. Vascular permeability was calculated by measuring the ΔR1 of tumors in control and treated animals at 4 hours post treatment.
Linear regression analysis of the normalized ΔR1 versus time curve was performed to compute the fractional blood volume (fBV) of tumors (22, 23). Values obtained at 24 hours were compared to baseline pretreatment estimates. T1 relaxation maps (R1 maps) of animals were calculated on a pixel-by-pixel basis in MATLAB (Math Works, Inc., Natick, MA, USA). For each treatment group, T1 enhancement maps (ΔR1 maps) were generated at baseline (pretreatment) and 24 hours post treatment by subtracting a post contrast R1 map from the precontrast R1 map of the same animal.

**Immunostaining for NG2 and CD31.** Twenty four hours after treatment, mice were anesthetized with ketamine (100 mg/kg; i.p.) plus xylazine (10 mg/kg; i.p.) and perfused with 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) at 2 mL/min using cardiac puncture of the left ventricle. After perfusion with fixative, tissue was dissected and immersed in 1% paraformaldehyde for 2 hours followed by immersion in 30% sucrose (Sigma-Aldrich) for 48 hours and embedded in OCT compound (Tissue-Tek, Sakura Finetek USA, Torrance, CA). OCT blocks were sectioned (6 μm) and slides were immersed in 1% BSA (Sigma-Aldrich) in PBS for 30 minutes. Sections were incubated overnight with the primary antibody, anti-NG2 Chondroitin Sulfate Proteoglycan (1/200, AB5320, Millipore, Billerica, MA) or anti-mouse CD31 (1/50, 550274, BD Biosciences, Franklin Lakes, NJ, USA). Slides were washed with PBS and sections were incubated with the secondary antibody FITC-conjugated anti-rabbit Ig (1/400, 554020, BD Biosciences) or Cy3-conjugated anti-rat IgG (1/400, A10522, Invitrogen, Carlsbad, CA). Sections were counterstained with DAPI and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Sections were visualized with a Zeiss Axioskop-2plus
microscope (Axioskop, Carl Zeiss). For NG2 and CD31 immunostaining quantification, 2-3 pictures of 3 samples per treatments were processed using Image J software to subtract background and to determine the integrated density of pixels. Results were expressed as the average per treatment of integrated density of pixels.

**Quantification of tumor necrosis.** Slides containing histologic sections of control and treated tumors were scanned and digitized using the Scanscope XT system (Aperio) and images of whole tumor sections were captured using the ImageScope software. Areas of necrosis were manually traced using the medical imaging software, Analyze (AnalyzePC; AnalyzeDirect, OverlandPark, KS) and reported as a percentage of the whole tumor area.

**Statistical analyses.** All measured values are reported as mean ± standard error. *P*-values < 0.05 were considered statistically significant. The two-tailed t test was used for comparing the individual treatment groups with the controls or combination treatment with the individual treatment groups at different times. Linear regression analysis of the change in R1 over time curve was performed to compute differences in fractional blood volume of tumors. All statistical calculations and analyses were done using GraphPad Instat (ver. 5.00, GraphPad Software, San Diego, CA).
RESULTS

Endothelial cell response to everolimus and ASA404 treatment in vitro

The three dimensional (3D) spheroid sprouting assay was performed to investigate the antiangiogenic/antivascular activity towards HUVECs of everolimus and ASA404 (Figure 1A and 1B respectively) individual treatments or in combination. As shown in Figure 1C (top panel), HUVECs underwent angiogenic mediated proliferation generating sprouts and formed new vessels in vitro in fibrin gel. After 24 hours of treatment with 100 μM ASA404, 1 nM everolimus or combination, the angiogenic potential of HUVEC spheroids was assessed. ASA404 or everolimus single treatments moderately inhibited HUVEC sprouting, while ASA404/everolimus combination displayed marked disruption of HUVEC sprouting ability (Figure 1C). Further assessment by staining HUVEC sprouts in 3-D culture with the DNA dye DAPI indicated that ASA404 single treatment did not induce significant loss in viable HUVEC sprouts ($p = 0.08$), whereas significant loss of HUVEC cell viability was found to be mediated by everolimus ($p = 0.03$). ASA404/everolimus combination treatment significantly increased disruption of HUVEC sprouts compared to ASA404 (*$p = 0.008$) and everolimus (**$p = 0.01$) single treatments (Figure 1D).

Combined mTOR inhibition and VDA treatment of IH RCC xenografts

To investigate the anti-tumor activity of ASA404 and everolimus in vivo, we treated nude mice bearing subcutaneous grafted human IH renal cell carcinoma xenografts with either everolimus (5 mg/kg; 5d on 2d off), ASA404 (25 mg/kg; once a week) or in combination for 35 days. All therapy groups demonstrated minimal toxicities as assessed by body
weight (data not shown). Tumor growth calculations and tumor weight measurements were obtained along with histological evaluation of tumor response to therapy. Figure 2A shows haematoxylin and eosin (H&E) stained tumor sections of control and treated IH xenografts. Tumors treated with ASA404 or everolimus showed moderate increases in amorphous hyaline deposits indicative of cellular necrosis compared to control treated tumors. In contrast, everolimus/ASA404 combination treatment of IH tumors resulted in marked induction of tumor hemorrhaging (He) and hyalinization (Figure 2A). A marked reduction in tumor weight was seen following combination treatment compared to controls. However, this reduction was not statistically significant compared to either monotherapy (Figure 2B). Combination treatment resulted in a significant delay in tumor growth compared to single treatments (Figure 2C; ASA404, \( p = 0.03 \) and everolimus \( p = 0.05 \)).

**Bioluminescence and MR imaging of orthotopic RENCA tumors**

We examined the antivascular and antitumor activity of ASA404/everolimus combination using the orthotopic RENCA tumor model. For these studies, a dual modality imaging based approach using bioluminescence imaging (BLI) and magnetic resonance imaging (MRI) was employed. Serial bioluminescence imaging was performed once every 3-4 days after orthotopic injection of luciferase-transfected RENCA cells to visualize successful tumor establishment in the kidneys based on bioluminescence signal (photon counts) as shown in Figure 3A. Approximately 2 weeks post implantation, non contrast-enhanced T2-weighted MRI was performed to confirm ‘tumor take’ and for assessment of tumor morphology and volume. Animals were then randomized into control or one of
the treatment groups. Figure 3A shows bioluminescence and MR images of a control mouse bearing orthotopic RENCA tumor in the right kidney (outlined in yellow). The left kidney is outlined in red on the MR images. MR images revealed invasive tumor growth which was confirmed by histologic examination (lower right panel).

**Vascular response to combined mTOR inhibition and VDA therapy in vivo**

Contrast enhanced MRI was performed to characterize the *in vivo* tumor vascular response of RENCA tumors to mTOR and VDA treatments when given alone and in combination. For these studies, mice with volume-matched tumors were studied (n=3 per group; mean tumor volume 87.12 ± 15.55 mm³).

We first examined treatment induced changes in vascular permeability by measuring the change in T1-relaxation rates (ΔR1) of RENCA tumors and contralateral kidneys (Fig 3B). Values obtained 4 hours post ASA404, everolimus or combination treatment were compared to untreated controls. Consistent with previous observations in subcutaneous tumor models, ASA404 treatment resulted in a significant increase (p < 0.05) in vascular permeability evidenced by increased accumulation of ΔR1 (0.578 ± 0.08) compared to controls (0.307 ± 0.04). While everolimus alone did not result in any change in vascular permeability (0.379 ± 0.16, p >0.05), tumors treated with the combination also showed an increase in ΔR1 (0.439 ± 0.04) compared to controls. However, this difference was not statistically significant (Figure 3B). No significant difference in contrast agent accumulation was observed in the contralateral kidneys of animals in the control and treatment groups. Figure 3C shows axial T2-weighted images and corresponding R1 maps of a representative mouse from each group at 4 hours post
treatment. Increased accumulation of the contrast agent can be visualized on the enlarged ROI of the tumor 4 hours post ASA404 treatment compared to untreated controls.

We next investigated the effects of ASA404, everolimus and combination treatment on fractional blood volume (fBV) of RENCA tumors. Figure 4 shows T1-enhancement maps of tumors at baseline (pre treatment) and 24 hours post treatment for all 3 treatment groups. Corresponding axial T2-weighted images are also shown for visualization of tumor extent (outlined in black). Reduction in contrast enhancement was seen 24 hours following treatment with ASA404 alone (Fig. 4A), everolimus alone (Fig. 4B), and combination (Fig. 4C) compared to baseline post contrast images. All 3 treatments resulted in a significant reduction in fBV at 24 hours (Fig. 4D). Treatment with ASA404 alone resulted in ~50% reduction fBV of RENCA tumors (0.207 ± 0.04; \( p<0.0001 \)) compared to pretreatment values (0.395 ± 0.02). Orthotopic RENCA tumors in animals treated with everolimus alone also exhibited a significant reduction in fBV (0.154 ± 0.01; \( p<0.001 \)) at the 24 hour time point compared to baseline estimates (0.289 ± 0.02). Combination treatment resulted in a similar reduction in fBV (0.165 ± 0.02; \( p<0.0001 \)) at the 24 hour time point compared to baseline measures (0.207 ± 0.04; \( p<0.0001 \)).

MRI based changes in vascular function were correlated with dual immunostaining of tumor sections for the pan endothelial cell adhesion molecule, CD31, and the pericyte marker, NG2. Histological assessment of tumor sections was also performed to visualize tumor necrosis following treatment. Consistent with the MRI results, CD31/NG2 staining revealed marked vascular damage following ASA404 alone and combination treatment (Figure 5A, bottom panel and Figures 5B and 5C). A
significant reduction ($p < 0.05$) in CD31 staining was seen following combination treatment compared to everolimus monotherapy, but not control treated animals ($p = 0.05$). Importantly, immunostaining demonstrated that either drug as single treatment or in combination was tumor specific, as staining of the non-tumor bearing contra-lateral kidney displayed no anti-vascular mediated effects (Figure 5A, top panel).

**Antitumor activity of ASA404/everolimus combination treatment of orthotopic RENCA tumors**

Finally, we examined the *in vivo* anti-tumor effects of ASA404, everolimus and combination treatment on orthotopic murine RENCA tumors. Overall, therapy was well tolerated with minimal toxicities exhibited as determined by body weight (data not shown). Therapeutic efficacy was determined by measurement of tumor weights and quantification of necrosis in histological sections in control and treated tumors. Figure 6A shows representative H&E sections of orthotopic RENCA tumors from control and treatment groups. Combination treatment with ASA404 and everolimus dramatically decreased the extent of this viable tumor rim compared to other treatment groups. A significant reduction ($p < 0.05$) in tumor weight was seen following combination treatment compared to controls and ASA404 treatment alone (Figure 6B).

Control tumors exhibited minimal amounts of tumor necrosis (9.2 ± 2.9%; Fig 6C). A significant increase in tumor necrosis was observed following treatment with ASA404 alone (46.0 ± 8.8%; $p < 0.01$) compared to untreated controls. Tumor necrosis following everolimus treatment was comparable to control tumors (6.3 ± 3.9; $p > 0.5$). Treatment with ASA404 alone resulted in marked necrosis of RENCA tumors, primarily
restricted to the central regions of the tumor with viable tumor cells visible in the rim. Maximal tumor necrosis was seen following combination treatment (81.0 ± 6.0%) in comparison to untreated controls \((p < 0.0001)\) and single agent therapy (ASA404, \(p = 0.02\) and everolimus, \(p = 0.0002\)).
DISCUSSION

Targeting the neo-vasculature in patients with advanced and/or metastatic renal cell carcinoma (RCC) has become the standard of care. The use of targeted therapies including angiogenesis inhibitors, TKIs and the mTOR inhibitors temsirolimus and everolimus has contributed to increased progression free survival and overall survival in RCC patients (8, 24, 25). However, these therapies are often not curative and a majority of patients develop recurrent disease. Therefore, investigation into novel treatment approaches that could improve treatment outcome in RCC is warranted.

Tumor VDAs represent a distinct class of drugs that target the established blood vessels of tumors and are actively being investigated for their therapeutic potential in preclinical studies and clinical trials in patients with solid tumors (12, 14, 15). Although the mechanism of action of these tumor-VDAs is not fully understood, agents such as ASA404 have been shown to exert both direct effects on the endothelium and indirect effects mediated by cytokines (17-19). Since angiogenesis inhibitors and VDAs target distinct vascular networks, combining VDAs with anti-angiogenic agents has been proposed as a novel strategy in the treatment of solid malignancies. In support of this argument, studies have previously reported enhanced antitumor activity with the combination of a VDA and antiangiogenic agents such bevacizumab against RCC (26, 27). In the present study, we investigated the activity of the tumor-VDA ASA404 in combination with the mTOR inhibitor everolimus.

Previous studies have demonstrated the antiangiogenic and antitumor properties of everolimus in vitro and in vivo (10, 28, 29). We have previously shown that mTOR inhibition with rapamycin in combination with the HDAC inhibitor panobinostat greatly
inhibited tumor angiogenesis by targeting HIF-1\(\alpha\) in endothelial cell lines (30). While everolimus and ASA404 have been shown to result in endothelial apoptosis when administered as single agents (10, 17), in the present study we observed enhanced inhibition of endothelial sprouting in 3D spheroid cultures. Combination of ASA404 and everolimus significantly enhanced cell death within endothelial cells.

The biological response of tumors to VDA treatment is typically characterized by early increases in vascular permeability followed by vascular collapse and cessation of blood flow leading to ischemia and tumor necrosis (18, 19, 21). In the present study, we utilized a dual modality imaging approach to examine the vascular response of orthotopic RENCA tumors to everolimus, ASA404 and combination treatment. While bioluminescence imaging enabled high-throughput visualization of tumor growth \textit{in vivo}, it has limited clinical applicability. Therefore, quantitative estimates of vascular permeability and perfusion were obtained following mono- and combination therapy using MRI. Contrast-enhanced MRI is one of the most widely used imaging methods for assessment of angiogenesis in preclinical studies and in patients enrolled in clinical trials (22, 23, 31, 32). Several studies have highlighted the usefulness of MRI methods in the assessment of tumor vascular response to antiangiogenic agents and VDAs (22, 32-34). MRI parameters of tumor vascularity are also being actively investigated for their utility as potential biomarkers of response in RCC patients (35-37). Consistent with previous observations in subcutaneous models (14, 15, 38), MRI detected an early and marked increase in vascular permeability following ASA404 treatment. Our MRI results also demonstrated a significant reduction in fractional blood volume of RENCA tumors after a single dose of ASA404, everolimus and combination treatment. It is therefore likely that
repeated doses of the VDA in conjunction with mTOR inhibition provide a cumulative assault on the tumor vascular network that results in catastrophic vascular damage subsequently leading to significant tumor ischemia. Our immunohistochemistry results provide supportive evidence of this increased tumor vascular damage following combination treatment. Consistent with the increased vascular damage, quantitative estimates of tumor necrosis were significantly greater with combination treatment compared to either monotherapy. Our in vivo studies carried out using IH RCC xenografts also demonstrated increased tumor growth inhibition following combination treatment.

Recently, Lara et al published results from a randomized phase III trial of NSCLC patients treated with chemotherapy with or without ASA404 (39). This recent phase III trial followed a promising phase II trial where the median overall survival (OS) for patients treated with chemotherapy and patients treated with chemotherapy with ASA404 was 8.8 and 14 months respectively (40). Unfortunately the phase III trial did not repeat the exciting results reported from the phase II trial. Overall survival for patients treated with chemotherapy and chemotherapy with ASA404 was 13.4 and 12.7 months respectively (39). While this data sheds an unfavorable light on the clinical development of VDAs, it highlights several issues relevant to the clinical development of VDAs. Given the temporal effects of VDAs such as ASA404 on tumor vascular function, optimization of the VDA dose and schedule becomes a critical issue when examining the clinical activity of combination strategies. It is likely that the interaction between VDAs and mTOR inhibitors or RTKs may be strongly influenced by the sequence or schedule of administration given that both agents exert effects on tumor vasculature. To date most
studies (including the present study) have been performed using concurrent administration of tumor-VDAs and RTKs or AIs. Alternative schedules or sequences warrant further investigation in order to develop a clinically-feasible yet optimized drug administration protocol for maximal therapeutic benefit. Additionally, lung and renal carcinomas have different biology, so unfavorable results towards the treatment of lung cancer patients does not mean ASA404 will necessarily fail in the treatment of patients with RCC.

Tumor-VDAs exhibit moderate activity as single agents and a classic observation seen in preclinical model systems is the presence of a surviving rim of viable tumor cells in the periphery (14, 15). The hypoxia induced within the tumor, as a consequence of tumor vascular disruption, results in the activation of hypoxia inducible factor 1α (HIF-1α) and increased expression of HIF-target genes including those involved in angiogenesis such as VEGF (41). This has been suggested as a possible escape mechanism of tumors following VDA treatment. Combining VDAs with inhibitors of the angiogenesis signaling pathways, such as the PI3K/Akt/mTOR pathways often activated by receptor tyrosine kinases (42) offers an alternative to target the viable tumor rim resistant to VDA therapy. Our observations highlight the potential of combined targeting of neo-vasculature and established vasculature of tumors as a promising new treatment approach for the management of RCC.

The mTOR inhibitor everolimus is an approved drug in patients with progressive RCC following treatment with VEGFR TKI (8). Results of the phase III trial in RCC patients revealed a median progression free survival of 5.1 months with everolimus compared to 1.9 months in the placebo arm (43). Despite this observed clinical benefit,
additional treatment in these patients is eventually necessary. To this end, therapeutic strategies simultaneously targeting the VEGF and the mTOR pathways to achieve greatest antitumor effect have been tested in preclinical models (28). Combinations of anti VEGF therapies and mTOR inhibitors have reported some clinical benefit but also increased toxicities as compared to single agents, resulting in significant dose reductions (44). Thus, the combination of a VDA and an mTOR inhibitor is also compelling in view of the lack of overlapping toxicities and the possibility of administering the two agents at doses associated with maximal antitumor activity. The disruptive effect of this combination on established blood vessels and in an orthotopic RCC model further suggests the potential use for this therapeutic strategy also on primary tumors in the neoadjuvant clinical setting. Given these advantages, clinical investigation into the combination of VDAs with mTOR inhibitors in RCC is warranted.
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REFERENCES


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FIGURE LEGENDS

Figure 1: Endothelial response to ASA404/everolimus treatment in vitro. (A)
Everolimus chemical structure. (B) ASA404 chemical structure. (C) Representative images of HUVEC spheroids either untreated (top panel) or treated with 100 μM ASA404, 1 nM everolimus or combination for 24 hours (bottom panel). (D) Treated spheroids from (A) were fixed and stained with DAPI to quantitate nuclear number and cell viability. Groups represent mean ±SE.

Figure 2: Response of IH RCC xenografts to VDA-mTOR inhibition in vivo. (A)
Photomicrographs of hematoxylin and eosin (H&E) stained sections of IH RCC xenografts obtained from animals in control and treatment groups. Magnification x40. Scale bars = 50 μm. (T) = tumor, (He) = hemorrhage. (B) Endpoint tumor weights; results for each treatment group represent mean ±SE (n=3). (C) Bi-weekly serial caliper measurements. Tumor size was calculated by LxW. Each treatment group was normalized to pre-treatment measurements and converted to percent tumor growth. Each point represents mean ±SE. * combination compared to single treatments (ASA404, p = 0.03 and everolimus p = 0.05).

Figure 3: Combined bioluminescence and MR imaging of orthotopic RENCA tumors. (A) Coronal and axial T2-weighted MR images (left) of a control mouse bearing orthotopic RENCA tumor in the right kidney (outlined in yellow). The contralateral kidney is outlined in red. Corresponding bioluminescence image of the same mouse is
also shown. Histologic examination confirmed the invasive pattern of tumor growth observed on imaging. (B). Bar graph shows ΔR1 measurements of tumor and contralateral kidneys at the 4 hour time point (n=3 per group) * indicates p <0.05 compared to controls. (C) Axial T2-weighted (T2W) MR images and corresponding R1 maps of a representative mouse from control and each of the treatment groups at 4 hours post treatment. An enlarged image of the tumor ROI is also shown.

**Figure 4: Vascular response to ASA404/everolimus combination in vivo.** Panel of images represent axial T2-weighted MR images and corresponding T1-enhancement (delta R1) maps of a mouse before (pretreatment) and 24 hours after a single treatment treated with ASA404 alone (A), everolimus alone (B) and combination (C). Post contrast images at 24 hours post treatment showed reduced enhancement compared to pretreatment images with all three groups (D) Bar graph shows fractional blood volume (fBV) measurements of tumors at the 24 hour time point (n=3 per group) *** indicates p <0.001 compared to baseline pretreatment values.

**Figure 5:** (A) Mice bearing orthotopic RENCA tumors and treated with 2.5 mg/kg everolimus, 22 mg/kg ASA404 or combination for 24 hours. OCT embedded kidney tissue samples were stained for CD31 (red) and NG2 (green). Top panel represents contra-lateral (control) kidney vasculature and bottom panel represents RENCA tumor tissue vasculature. (B) Quantitation of CD31 and NG2 fluorescence intensity RENCA tumor tissue.
**Figure 6:** (A) Four micron RENCA tumor sections were stained with hematoxylin and eosin for microscopic examination. Magnification x20 (left panels) x40 (right panels). Scale bars = 50 µm. (T) = tumor, (NK) = normal kidney, (N) = necrosis (plus short arrows). Long arrows in combination panel indicate viable tumor rim (B) Endpoint tumor weights; results for each treatment group represent mean ±SE (n=3). ns = not significant, *p < 0.0001. (C) Tumor necrosis was quantitated as described (see Materials and Methods). Each point represents mean ±SE. ASA404, *p = 0.02 and everolimus **p = 0.0002).
Figure 2

A

Control

Everolimus

A8304

Combination

B

Tumor Weight (g)

Control

A8304

Everolimus

Combination

ns

C

Relative tumor growth (%)

Control

A8304

Everolimus

Combination

Days on treatment

0

100

200

300

400
Figure 5

A  Vehicle  Everolimus  ASA404  Combination

B  CD31

C  NG2
Figure 6

A

B

Tumor Weight (g)

Control
Alka4
Evolimus
Combination

ns

C

% Necrosis

Control
Alka4
Evolimus
Combination