Vorinostat Enhances the Activity of Temsirolimus in Renal Cell Carcinoma Through Suppression of Survivin Levels

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Abstract

Purpose: The mammalian target of rapamycin (mTOR) inhibitor temsirolimus has exhibited promising anticancer activity for the treatment of renal cell cancers (RCC). Survivin expression has been implicated in drug resistance and reducing its levels with the histone deacetylase (HDAC) inhibitor vorinostat may enhance the anticancer activity of temsirolimus.

Experimental Design: The sensitivity of RCC cell lines to the combination of temsirolimus and vorinostat was determined by measuring cell viability, clonogenic survival, and apoptosis. The effects of this combination on survivin levels were determined in vitro and in vivo. Survivin expression was silenced using small interfering RNA to evaluate its role in determining sensitivity to temsirolimus and vorinostat. The effect of the combination on angiogenesis was also determined in RCC xenograft models.

Results: Vorinostat synergistically improved the anticancer activity of temsirolimus in a panel of RCC cell lines in vitro and in two xenograft models in vivo. While each single agent led to a modest decrease in survivin levels, the combination dramatically reduced its expression, which correlated with an induction of apoptosis. Silencing survivin levels induced apoptosis and significantly improved the efficacy of temsirolimus and vorinostat. In addition, the temsirolimus/vorinostat combination led to a strong reduction in angiogenesis.

Conclusions: Vorinostat augmented the anticancer activity of temsirolimus in both in vitro and in vivo models of RCC. The effectiveness of the combination was due to a decrease in survivin levels and corresponding induction of apoptosis, and enhanced inhibition of angiogenesis. Targeting survivin may be a promising therapeutic strategy to improve RCC therapy. Clin Cancer Res; 16(1); 141–53. ©2010 AACR.

Renal cell carcinoma (RCC) is one of the most lethal genito-urinary malignancies with about 13,000 estimated cancer-related deaths in the United States in 2008 (1). New treatment options for this disease including the Food and Drug Administration (FDA)-approved agents temsirolimus, everolimus, sorafenib, and sunitinib have improved clinical outcomes. Temsirolimus and everolimus are inhibitors of mammalian target of rapamycin (mTOR) and sorafenib and sunitinib are multi-tyrosine kinase inhibitors. The success of these agents has largely been attributed to their ability to disrupt angiogenesis, which plays a critical role in the development and progression of RCC (2, 3). While the use of these drugs has improved clinical response rates, drug resistance is an emerging problem. mTOR is a critical regulator of cellular protein synthesis that influences many aspects of tumor proliferation, including cell cycle progression, angiogenesis, and apoptosis and functions as a downstream effector of the essential phosphatidylinositol 3-kinase (PI3K)-Akt cell survival pathway that is deregulated in many cancers (4). Activation of mTOR stimulates translation of oncopgenic proteins such as c-Myc and hypoxia-inducible factor 1α (HIF-1α), which results in an increase in genes that promote angiogenesis (vascular endothelial growth factor (VEGF), proliferation (cyclin D1), and cell survival (survivin) (refs. 5–7). Aberrant PI3K/AKT signaling via enhanced growth factor stimulation, mutation or amplification, and loss or downregulation of the PTEN tumor suppressor gene leads to increased mTOR activation (8).

As a component of an E3 ubiquitin ligase, von Hippel Lindau (VHL) targets HIF-1α and HIF-2α for proteasomal degradation (9). Loss of VHL function prevents the degradation of these factors, leading to increased expression of HIF-regulated genes and increased angiogenesis (10). In sporadic RCC, loss of function of the VHL tumor suppressor gene has been well documented (11). Consistent with this molecular profile, RCC is a highly vascular disease, which may explain why these tumors are especially susceptible to temsirolimus and other antiangiogenic therapies (12). Despite the success of temsirolimus, most...
Collectively, these results establish that survivin contributes to temsirolimus, vorinostat, and the combination. In addition, overexpression of survivin levels conferred resistance to temsirolimus and vorinostat alone stimulated only modest levels of apoptosis, the combination led to enhanced disruption of angiogenesis compared to single agent treatment. These data demonstrate that the temsirolimus/vorinostat combination has significant activity in RCC and targeted disruption of survivin levels may help sensitize tumors to mTOR or HDAC inhibitor-mediated cell death.

The HDAC inhibitor vorinostat was recently FDA-approved for the treatment of renal cell cancer (RCC) and targeted disruption of survivin levels may help sensitize tumors to mTOR or HDAC inhibitors may have activity in this cancer type (19).

Deacetylation of histones is associated with transcriptional repression, including a decrease in the expression of tumor suppressor genes. Since abrogation of histone deacetylase (HDAC) activity can reverse the epigenetic silencing that is frequently observed in cancer, various HDAC inhibitors have been developed for cancer therapy (13). The HDAC inhibitor vorinostat was recently FDA-approved for cutaneous T-cell lymphoma and is under investigation for the treatment of other malignancies (14). These inhibitors have been shown to induce cell cycle arrest, apoptosis, and similar to temsirolimus, also have been reported to disrupt angiogenesis (15–18). A recent study determined that HDACs are highly expressed in RCC, and thus, these inhibitors may have activity in this cancer type (19).

In this report, we show that the antinecancer activity of temsirolimus can be enhanced by vorinostat in both in vitro and in vivo models of RCC. The combination led to a significant decrease in angiogenesis and tumor cell proliferation compared to either single agent treatment. While temsirolimus or vorinostat alone stimulated only modest levels of apoptosis, the combination resulted in a substantial increase in cell death. Therefore, the efficacy of the combination is likely due to the cooperative effects of these agents on angiogenesis, proliferation, and apoptosis. The onset of apoptosis correlated with a dramatic decrease in survivin levels, which was further validated using RNA interference. In addition, overexpression of survivin levels conferred resistance to temsirolimus, vorinostat, and the combination. Collectively, these results establish that survivin contributes to temsirolimus resistance in RCC, which may be overcome via simultaneous inhibition of mTOR and HDACs.

Materials and Methods

Cells and cell culture. 786-O, A498, 769-P, Caki-1, Caki-2, SW839, ACHN, G401 and SK-NEP-1 renal cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Human renal cancer cell lines were maintained in RPMI supplemented with 10% fetal bovine serum in a humidified incubator at 37°C with 5% CO2.

Antibodies and reagents. Antibodies were obtained from the following commercial sources: anti-survivin, VEGF, XIAP, and HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA); anti-tubulin (Sigma-Aldrich, St Louis, MO); anti-HIF-2α (Novus, Littleton, CO); CD31-platelet/endothelial cell adhesion molecule 1, bcl-2, and bcl-xL (PharMingen, San Diego, CA), anti-proliferating cell nuclear antigen (PCNA) (Dako, Glostrup, Denmark); goat anti-rabbit and goat anti-rat horseradish peroxidase (HRP)-conjugated secondary antibodies and goat anti-rat Texas Red (Jackson Laboratories, West Grove, PA). Rat anti-mouse IgG2a-HRP (Serotec, Raleigh, NC), and sheep anti-mouse-HRP and donkey anti-rabbit-HRP (Amersham, Pittsburgh, PA). Temsirolimus and vorinostat were purchased from the CTRC pharmacy.

Quantification of drug-induced cytotoxicity. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were seeded into 96-well microculture plates at 10,000 cells per well and allowed to attach for 24 hours. Cells were then treated with temsirolimus, vorinostat or the combination for 72 hours. Following drug treatment, MTT was added and cell viability was quantified using a BioTek (Winooski, VT) microplate reader. Pro-apoptotic effects following in vitro drug exposure were quantified by propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis of sub-Go/G1 DNA content as previously described (20).

Preparation and transfection of survivin siRNA and overexpression constructs. Survivin SMARTpool and non-target siRNA were obtained from Dharmacon (Lafayette, CO). Cells were transfected with 100 nM of each siRNA using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Transfected cells were incubated at 37°C for 24 hours without changing the medium. Cells were then treated with drugs for 72 hours. Efficiency of RNAi was measured at 72 hours by immunoblotting using an anti-survivin antibody. Survivin overexpression was carried out using the pORF5-hSurvivin expression vector (Invitrogen, San Diego, CA). Lyophilized GT100 E. coli bacteria transformed by pORF5-hSurvivin and pORF-mcs were streaked on an E. coli Fast-Media Amp agar plate (Invitrogen, San Diego, CA). After incubation at 37°C overnight, colonies from each construct were grown in LB supplemented with ampicillin using the Fast-Media Amp liquid (Invitrogen, San Diego, CA). Plasmids were isolated using a Qiagen mini-prep plasmid isolation kit (Qiagen Inc, Valencia, CA). 786-O cells were transiently

Translational Relevance

Inhibitors of mammalian target of rapamycin (mTOR), including temsirolimus have efficacy in the treatment of renal cell cancer (RCC) and are currently under investigation in other malignancies. Despite the initial success of this class of agents, drug resistance continues to be a major obstacle. In the current study, the histone deacetylase (HDAC) inhibitor vorinostat enhanced the anticancer activity of temsirolimus in multiple RCC models. The anti-neoplastic mechanism of this combination appears to be multifaceted. Combination treatment led to a strong reduction in survivin levels, which was associated with the induction of apoptosis and a reduction in tumor proliferation. In addition, the combination led to enhanced disruption of angiogenesis compared to either single agent treatment. These data demonstrate that the temsirolimus/vorinostat combination has significant activity in RCC and targeted disruption of survivin levels may help sensitize tumors to mTOR or HDAC inhibitor-mediated cell death.
transfected with pORF5-hSurvivin and the empty vector pORF- 
mcs using the TransFast reagent (Promega, Madison, WI) 
according to the manufacturer’s instructions. Briefly, 1 μg of 
each plasmid was diluted in serum-free medium. The 
diluted plasmids were vortexed before the addition of 3 μL Transfast reagent to bring the Transfast/DNA ratio to 1:1. DNA/Transfast mixtures were incubated at room temperature for 30 min to allow the Transfast/DNA complex formation. Cells were washed once with serum-free medium before adding DNA/Transfast mixtures. Cells in Transfast/DNA mixture were then incubated at 37 °C for 1 hour. Following this incubation period, serum-containing medium was added to each well, and cells were incubated at 37 °C overnight. Cells were then treated with drugs for 72 hours and apoptosis was measured by PI-FACS analysis. Survivin overexpression was confirmed at 72 hours by immunoblotting.

Clonogenic survival assays. Cells were treated with the indicated concentrations of temsirolimus, vorinostat, or the combination for 72 hours. Drug-treated cells were then washed twice in PBS followed by the addition of fresh media. The cells were incubated for 10 days in a humidified incubator at 37 °C with 5% CO₂. Colonies were washed in PBS, fixed with methanol, and stained with crystal violet. Colonies were scored using an Alpha Innotech (San Leandro, CA) gel documentation system. The dose-response curves of each agent for each cell line were obtained. Nine combination treatment groups were evaluated and the interactions between drugs were determined by calculating Chou-Talalay combination indices (CI) using CompuSyn software (ComboSyn, Inc, Paramus, NJ). Each condition was replicated in triplicate.

Immunoblotting. Renal cancer cells were incubated with 10 μM temsirolimus, 2 μM vorinostat or the combination for 48 h. Cells were collected using a cell scraper at 4°C and were then lysed as previously described (21). Approximately 50 μg of total cellular protein from each sample were subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and blocked with 5% nonfat milk in a Tris-buffered saline solution containing 0.1% Tween-20 for 1 hour. The blots were then probed overnight with the relevant primary antibodies, washed, and probed with species-specific secondary antibodies coupled to HRP. Immunoreactive material was detected by enhanced chemiluminescence (West Pico, Pierce, Inc., Rockville, IL).

Xenograft studies. 786-O and Caki-1 renal cancer cells (1 x 10⁇) were harvested, washed in PBS, and suspended in a mixture of HBSS and Matrigel (BD BioSciences, San Jose, CA). Cells were then subcutaneously implanted into female nude mice (BALB/c background) from Harlan (Indianapolis, IN). Tumor-bearing animals from each cell line xenograft were randomized into treatment groups. Mice were treated with vehicle, temsirolimus (10 mg/kg IV), vorinostat (100 mg/kg PO), or both agents on a QDx5 (every day for 5 days) schedule for 3 weeks. Mice were monitored daily and tumor volumes were measured twice weekly. At the completion of the study, tumors were excised, formalin-fixed and paraffin-embedded for immunohistochemical analysis.

Immunohistochemistry. Paraffin-embedded tumor sections (4-6 μm thick) were mounted on slides, which were used to determine VEGF, PCNA, TUNEL, and survivin expression levels. Sections were deparaffinized in xylene, treated with a graded series of alcohol [100%, 95%, and 80% ethanol/double-distilled H₂O (v/v)] and rehydrated in PBS (pH 7.5). Heat-induced epitope retrieval was performed by microwaving slides in a citrate buffer for 5 minutes. The slides were allowed to cool and endogenous peroxidases were blocked with a 3% hydrogen peroxide solution for 10 minutes. Slides were then incubated in a protein block solution (5% horse and 1% goat serum in PBS) for 20 minutes. Primary antibodies were diluted in the protein block solution and placed at 4°C overnight. After washing with PBS, slides were incubated in appropriate secondary antibodies for 1 hour at ambient temperature. Positive reactions were visualized by immersing the slides with stable 3,3'-diaminobenzidine diamnobenzidine (Research Genetics, Huntsville, AL) for 10-20 min. The sections were rinsed with distilled water, counterstained with Gill's hematoxylin (Sigma, St. Louis, MO), and mounted with Universal Mount (Research Genetics, Huntsville, AL). For CD31 and CD31-TUNEL staining, frozen tumor sections were used and slides were fixed with acetone and chloroform. Images were captured using an Olympus fluorescent microscope (Center Valley, PA) with a DP71 camera and a 20× objective. Image-Pro Plus software Version 6.2.1 (Media Cybernetics, Bethesda, MD) was used for image acquisition and quantification by densitometric analysis of five random high-power fields containing viable tumor cells. Quantification of microvessel density (MVD) and PCNA was conducted by counting the number of positive structures in five random fields.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling assay. DNA fragmentation was analyzed using a FITC-labeled terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay kit (Promega, Madison, WI). The assay was carried out according to the manufacturer’s instructions. Propidium iodide was used to counterstain the nucleus. All slides were mounted using Prolong anti-fade reagent (Molecular Probes, Eugene, OR). Images were obtained with an Olympus fluorescent microscope (Center Valley, PA) with a DP71 camera and a 20× objective. Percentages of TUNEL-positive cells were determined by manual counting of 5 random fields per section. Image-Pro Plus software Version 6.2.1 (Media Cybernetics, Bethesda, MD) was used for image acquisition. For CD31 TUNEL staining, frozen sections were stained using an anti-CD31 primary antibody followed by a Texas red secondary antibody. TUNEL analysis was then performed as described above.

Statistical analyses. Statistical significance of differences observed between samples was determined using the Tukey-Kramer Comparison Test or the Student’s t test. Differences were considered significant in all experiments at P < 0.05.
Fig. 1. Vorinostat enhances the anticancer activity of temsirolimus, which is correlated with a reduction in survivin expression. A, vorinostat and temsirolimus reduce RCC cell viability. Nine renal cancer cell lines were incubated with 10 μM temsirolimus, 2 μM vorinostat, or the combination for 72 h and cell viability was measured by MTT assay. Mean ± SD, n = 3. *Indicates a significant difference compared to either single agent treatment. P < 0.05.

B, the combination of temsirolimus and vorinostat strongly reduce the clonogenic survival of RCC cells. Cells were treated with 50 nM temsirolimus, 2 μM vorinostat, or both agents for 72 h. Cells were then washed and incubated in fresh medium for 10 d. Colonies were stained and scored as described in Materials and Methods. Mean ± SD, n = 3. *Indicates a significant difference compared to controls and **indicates a significant difference compared to either single agent treatment. P < 0.05.

C, vorinostat augments temsirolimus-mediated apoptosis. RCC cells were treated with 10 μM temsirolimus, 2 μM vorinostat, or both agents for 48 h. The effects of the drugs on cell cycle and apoptosis were determined by PI staining and flow cytometry. D, the temsirolimus/vorinostat combination induces a potent decrease in survivin levels. 786-O and Caki-1 cells were treated with 10 μM temsirolimus, 2 μM vorinostat, or both agents for 48 h. Protein expression levels were determined by immunoblotting as described in Materials and Methods. A decrease in survivin levels correlates with drug-induced apoptosis as measured by PI-FACS. Mean ± SD, n = 3. *Indicates a significant difference compared to controls and **indicates a significant difference compared to either single agent treatment. P < 0.05.
Fig. 2. Modulation of survivin levels alters the sensitivity of RCC cells to temsirolimus- and vorinostat-induced cell death. A, silencing survivin expression using siRNA. RCC cells were transfected with non-target (NT) or survivin siRNA as described in Materials and Methods and immunoblotting confirmed a reduction in survivin levels at 72 h. B, knockdown of survivin levels enhances the anticancer activity of temsirolimus and vorinostat. Cells were either untransfected or treated with NT or survivin siRNA and then treated with the indicated concentrations of temsirolimus or vorinostat for 72 h. Cell viability was measured by MTT assay. Mean ± SD, n = 3. *Indicates a significant difference compared to the NT transfected cells treated with the same concentration. P < 0.05. C, knockdown of survivin enhances temsirolimus- and vorinostat-induced apoptosis. 786-O and Caki-1 cells transfected with NT or survivin siRNA were treated with 10 μM temsirolimus, 2.5 μM vorinostat, or the combination for 72 h. Apoptosis was determined by PI staining and flow cytometry. Mean ± SD, n = 3. *Indicates a significant difference compared to the NT transfected cells treated with the same concentration. P < 0.05. D, transient overexpression of survivin reduces temsirolimus- and vorinostat-induced apoptosis. 786-O cells were transfected with pORF-mcs (vector containing a multiple cloning site) or pORF-Survivin as described in Materials and Methods. Immunoblotting confirmed survivin overexpression at 72 h. Cells were treated with 10 μM temsirolimus, 2.5 μM vorinostat, or the combination for 72 h. Apoptosis was determined by PI-FACS. Mean ± SD, n = 3. *Indicates a significant difference compared to pORF-mcs transfected cells treated with the same concentration. P < 0.05.
Results

Vorinostat enhances the activity of temsirolimus in RCC. Although temsirolimus has shown clinical benefit in RCC, drug resistance continues to be a major problem (22). HDAC inhibitors have a multifaceted mechanism of action, which includes the induction of oxidative stress, apoptosis, growth arrest, and inhibition of angiogenesis (14). Considering this, we hypothesized that the HDAC inhibitor vorinostat would augment the anticancer activity of temsirolimus in both in vitro and in vivo models of RCC.

To test this hypothesis, nine RCC cell lines were treated with temsirolimus, vorinostat, or the combination of these agents for 72 hours and the cytotoxic effects were measured by MTT assay. Temsirolimus produced a modest decrease in cell viability in the majority of the cell lines tested; however the addition of vorinostat significantly improved its anticancer activity (Fig. 1A). Clonogenic survival assays were conducted to evaluate the prolonged effects of low dose temsirolimus and vorinostat on growth and survival. Formal synergy analysis determined that combinations of 50 nM temsirolimus and 1 μM vorinostat combined with various doses of vorinostat (50 nM - 5 μM) or temsirolimus (10 nM - 1 μM) all produced combination indices (CI) lower than 1 (range = 0.1 - 0.9), thus demonstrating the synergistic anticancer activity of this combination. Figure 1B shows the clonogenic survival results of the combination of 50 nM temsirolimus and 1 μM vorinostat. We next investigated the effects of the temsirolimus/vorinostat combination on cell cycle progression and apoptosis. PI/FACS analysis showed that temsirolimus stimulated a marginal increase in apoptosis, however co-treatment with vorinostat significantly increased cell death compared to either single agent (Fig. 1C and D). Survivin is a key regulator of the mitotic spindle checkpoint, an inhibitor of apoptosis, and has recently been identified as a chemoresistance factor (23–25). Furthermore, high survivin expression has recently been correlated with aggressive disease and a poor prognosis in RCC (26, 27). Immunoblotting was performed to evaluate its expression following drug treatment. Both temsirolimus and vorinostat partially decreased survivin levels, however the combination potently reduced protein expression (Fig. 1D). Notably, temsirolimus and vorinostat did
not significantly alter the expression of other key survival factors (Bcl-2, Bcl-xL, and XIAP) (Fig. 1D). These data suggest that a reduction in survivin levels may sensitize RCC cells to apoptosis induced by temsirolimus or vorinostat.

Survivin expression promotes drug resistance to temsirolimus and vorinostat. To determine whether survivin expression modulates apoptosis induced by temsirolimus or vorinostat, RNA interference was used to silence its expression in the 786-O and Caki-1 cell lines (Fig. 2A). MTT assays showed that knockdown of survivin enhanced the anticancer activity of temsirolimus and vorinostat (Fig. 2B). Furthermore, silencing of survivin levels also sensitized both renal cancer cell lines to temsirolimus- and vorinostat-mediated apoptosis (Fig. 2C). To further investigate the role of survivin in stimulating resistance to temsirolimus and vorinostat-mediated apoptosis, we transiently overexpressed survivin in 786-O RCC cells. As expected, higher levels of survivin led to a significant decrease in apoptosis induced by both single agents and the combination (Fig. 2D). Collectively, these data demonstrate that targeting survivin expression may be a viable strategy to enhance the anticancer activity of temsirolimus and vorinostat.

Vorinostat cooperates with temsirolimus to reduce tumor burden in RCC xenografts. Temsirolimus did not exhibit strong in vitro activity when evaluated in a panel of RCC cell lines (Fig. 1A). Of the nine renal cancer lines tested, only the two Wilm's tumor lines (G401 and SK-NEP-1) possessed an IC\textsubscript{50} < 20 \textmu M in 72-hour MTT assays (data not shown). However, the agent was more potent at reducing RCC viability in clonogenic survival assays, which is likely a better method of evaluating its activity (Fig. 1B). Considering the pleiotropic effects of temsirolimus, its activity is probably best evaluated using in vivo models. To further investigate the potential benefit of the temsirolimus and vorinostat combination, xenograft studies were performed. 786-O and Caki-1 cells were implanted into nude mice. Tumor-bearing animals were randomized into groups and given 10 mg/kg temsirolimus, 100 mg/kg vorinostat, or the combination for 3 weeks on a QDX5 schedule. Treatment with temsirolimus alone resulted in a dramatic decrease in mean tumor volume in 786-O and Caki-1 tumors compared to the vehicle treated controls (Fig. 3A and B). Vorinostat stimulated a moderate reduction in tumor growth in both models, but significantly enhanced the efficacy of temsirolimus in both 786-O and Caki-1 models (Fig. 3A and B). In addition, all drug treatments were very well tolerated, as no significant animal weight loss was observed in either study (Fig. 3C and D). The reasons for the differential potency of temsirolimus in vivo versus in vitro are not entirely clear, but may be due to the strong anti-angiogenic effects previously reported following mTOR inhibitor treatment (28, 29).

Angiogenesis is impaired by the temsirolimus/vorinostat combination in RCC. Angiogenesis is a critical component of tumor progression and is thus an important target in cancer therapy. Since we observed a strong antitumor response in two RCC xenograft models, we evaluated the effects of temsirolimus and vorinostat on angiogenesis by measuring HIF-1\textalpha and HIF-2\textalpha levels, intratumor vasculature (using CD31 as an endothelial cell marker), endothelial cell death (using CD31-TUNEL staining), and VEGF expression. HIF-1\textalpha was not detected by immunoblot in these cell lines (data not shown), which is consistent with a prior study (30). However, both cell lines expressed HIF-2\textalpha. As expected, HIF-2\textalpha levels were higher in the VHL-deficient 786-O cells compared to Caki-1 (Fig. 4A). Temsirolimus and vorinostat led to a minor decrease in HIF-2\textalpha levels and importantly the combination of the two drugs produced an enhanced reduction in HIF-2\textalpha expression (Fig. 4A). The combination also reduced the number of vessels in both 786-O and Caki-1 tumors (Fig. 4B) and led to a significant increase in endothelial cell death (Fig. 4C). Notably, the temsirolimus/vorinostat combination resulted in a dramatic decrease in the size and number of vessels compared to either single agent treatment (Fig. 4B and C). Consistent with the observed decrease in vessel density, the combination also produced significant reductions in VEGF in both xenograft models (Fig. 4D). These results suggest that enhanced disruption of angiogenesis may significantly contribute to the efficacy of the temsirolimus/vorinostat combination.

Temsirolimus and vorinostat inhibit tumor cell proliferation and stimulate apoptosis. Immunohistochemical analyses were performed to determine the effects of the temsirolimus/vorinostat combination on tumor cell proliferation and apoptosis. Paraffin-embedded tumor sections were stained with an anti-PCNA antibody to monitor proliferation. Both single agents decreased the percentage of PCNA-positive cells; however the combination further reduced tumor proliferation compared to mice treated with temsirolimus or vorinostat alone (Fig. 5A and B). To determine the ability of these agents to induce apoptosis, TUNEL assay was performed on the tumor sections. Both single agents alone produced very modest proapoptotic effects; however the combination further reduced tumor proliferation compared to mice treated with temsirolimus or vorinostat alone (Fig. 5A and B). Taken together, this data demonstrates that the temsirolimus/vorinostat combination suppresses cytostatic and cytotoxic activity in RCC.

Suppression of survivin levels correlates with temsirolimus/vorinostat-mediated apoptosis. Our xenograft studies demonstrate that vorinostat augments the anticancer activity of temsirolimus and this effect is associated with a decrease in tumor cell proliferation and an induction of apoptosis. Since survivin is a key regulator of cell division and apoptosis and has been linked to drug resistance, we determined its expression in these RCC models. Immunohistochemistry revealed that survivin levels were significantly reduced in tumors stained from temsirolimus and vorinostat treated mice (Fig. 6A and B). However, the combination of both agents produced a further decrease in survivin expression (Fig. 6A and B), which may have led to a corresponding increase in tumor cell apoptosis (Fig. 5C and D). Thus, the temsirolimus/vorinostat combination demonstrates potent antitumor activity in RCC.
which is associated with a major reduction in survivin expression.

**Discussion**

Both the incidence and mortality due to RCC have been on the rise over the last several decades (1). Recently, there are new treatment options for the therapy of RCC: mTOR inhibitors (temsirolimus and everolimus) and multi-tyrosine kinase inhibitors (sunitinib and sorafenib) (ref. 3). Since RCC is a highly vascular disease, it is an attractive approach to target angiogenesis for treatment of this malignancy (11). However, drug resistance continues to limit the efficacy of these agents. Therefore, novel strategies are desperately needed to overcome resistance and improve clinical outcomes. HDAC inhibitors have been shown to induce apoptosis, decrease tumor growth and inhibit angiogenesis (31–34). In addition, the utility of HDAC inhibitors is further enhanced by their ability to synergize with...
**Fig. 4 Continued.**

C, CD31-TUNEL immunohistochemistry. Frozen tumors were stained with an anti-CD31 antibody followed by a Texas Red-conjugated fluorescent secondary. TUNEL was then performed to visualize apoptosis. Vessels that were TUNEL-positive were manually counted in random fields. Mean ± SD, n = 5. *Indicates a significant difference compared to controls and **indicates a significant difference compared to either single agent treatment. P < 0.05.

D, VEGF immunohistochemistry. Sections were stained with an anti-VEGF antibody and a nuclear counterstain. The relative intensity of VEGF expression was measured using Image-Pro Plus software Version 6.2.1. Mean ± SD, n = 5. *Indicates a significant difference compared to controls and **indicates a significant difference compared to either single agent treatment. P < 0.05.
Fig. 5. Temsirolimus and vorinostat inhibit proliferation and induce apoptosis in RCC xenografts. A, PCNA immunohistochemistry. Tumors were stained with an anti-PCNA antibody as described in Materials and Methods. B, quantification of PCNA-positive cells. Stained cells were scored manually. Mean ± SD, n = 5. *Indicates a significant difference compared to controls and **indicates a significant difference compared to either single agent treatment. P < 0.05. C, apoptosis was determined by TUNEL assay. TUNEL staining was carried out as described in Materials and Methods. D, quantification of TUNEL-positive cells. Positive cells were scored manually under 20× magnification. Mean ± SD, n = 5. *Indicates a significant difference compared to controls and single agent treatments. P < 0.05.
many anticancer agents, including mTOR inhibitors (35–37).

Our data demonstrates that enhanced inhibition of angiogenesis by the temsirolimus/vorinostat combination likely contributes to the activity of these agents in xenograft models. Consistent with this hypothesis, a recent study showed that the combination of the mTOR inhibitor rapamycin and the HDAC inhibitor LBH589 significantly reduced HIF-1α levels and vessel density (36). The Caki-1 and 786-O RCC cell lines were selected for in vivo evaluation based on their differing VHL status. As expected, the VHL−/− 786-O cell line possessed higher levels of VEGF compared to the VHL+/+ Caki-1 (Fig. 4C). Despite the important role that VHL plays in RCC, the temsirolimus/vorinostat combination induced a strong antitumor response in both xenograft models. Our data suggests that this therapeutic approach may be equally effective regardless of VHL status.

While mTOR and HDAC inhibitors can disrupt angiogenesis, both classes of agents have also been reported to induce cell cycle arrest. The anti-proliferative effect of mTOR inhibitors may be attributed to their ability to downregulate synthesis of essential cell cycle proteins, including cyclin D1, HIF-1α, and c-Myc (4, 22). Similarly, HDAC inhibitors have been shown to induce p21 and stimulate cell cycle arrest (18). Consistent with these

![Fig. 6.](image-url)
reports, both agents reduced tumor cell proliferation. However, the co-administration of temsirolimus and vorinostat led to a dramatic reduction in PCNA-positive cells, suggesting that this combination has potent cytostatic effects.

In our study, mTOR inhibitors displayed limited pro-apoptotic effects, but were rather potent inhibitors of proliferation and angiogenesis. This observation may also explain, in part, the paradoxical result of why mTOR inhibitors are much more efficacious in vivo versus in vitro (29). However, the lack of cytotoxicity of mTOR inhibitors may ultimately limit their utility as anticancer agents. On the other hand, HDAC inhibitors have been well documented to induce apoptosis in many tumor types (15, 32, 38, 39). The pro-apoptotic nature of HDAC inhibitors is related to their ability to upregulate apoptotic-promoting molecules such as TRAIL, Fas, and Bim, hyperacetylate and stabilize p53, induce oxidative stress, and downregulate inhibitors of apoptosis (14, 40). Importantly, our study demonstrates that vorinostat enhances the ability of temsirolimus to promote apoptosis.

Apoptosis induced by the temsirolimus/vorinostat combination was associated with a highly significant decrease in survivin levels. Survivin has multiple functions and regulates both cell division and cell survival (23–25). It was initially identified as a member of the inhibitor of apoptosis (IAP) family that structurally contains a single BIR (baculoviral IAP repeats) domain, which is shared by other members, such as XIAP, c-IAP1, c-IAP2 (41, 42). While the specific mechanism is controversial, survivin has been shown to inhibit apoptosis in a number of models (32, 43). In our study, we show that both temsirolimus and vorinostat stimulate a moderate reduction in survivin levels individually, but the combination results in a potent decrease in its expression. This effect on survivin was detected in both in vitro and in vivo RCC models and was associated with an increase in apoptosis. Furthermore, targeted knockdown of survivin sensitized RCC cells to temsirolimus and vorinostat-mediated apoptosis and conversely, survivin overexpression blunted the apoptotic response. We also attempted to stably knockdown survivin in 786-O cells using shRNA lentiviral constructs to evaluate the effects of survivin deficiency on tumor growth and sensitivity to temsirolimus and vorinostat in a xenograft model. 786-O cells lacking survivin displayed markedly attenuated growth potential in vitro and failed to establish tumors in vivo. Our results are consistent with reports from other investigators demonstrating that survivin-deficient cells have low tumor formation in xenografts (44, 45). Taken together, these results suggest that survivin is a key regulator of RCC biology that is essential for tumor progression.

Increased survivin expression is an unfavorable prognostic marker associated with decreased overall survival in many malignancies, including renal, colon, and breast cancer (26, 27, 41). Considering the pleiotropic role that survivin plays in cell proliferation and apoptosis, it is very promising therapeutic target. A number of small molecule inhibitors of survivin, such as YM155 are in early clinical development (25, 46). Collectively, our findings demonstrate that vorinostat augments the anticancer activity of temsirolimus in RCC through enhanced inhibition of angiogenesis and tumor cell proliferation and stimulation of apoptosis via downregulation of survivin expression (Fig. 6C). Continued clinical investigation of the temsirolimus/vorinostat combination is particularly warranted as it was very well tolerated in vivo. This study provides a strong rationale for the design of clinical trials to evaluate the efficacy of vorinostat in combination with temsirolimus in RCC and would encourage a focus on targeting survivin in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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