

Advanced retinoblastoma treatment: targeting hypoxia by inhibition of the mammalian target of rapamycin (mTOR) in LH_{BETA}T_{AG} retinal tumors

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Purpose: The purpose of this study is to analyze the dose response of the mammalian target of rapamycin (mTOR) inhibitor, rapamycin, on tumor burden and hypoxia, and study the treatment effect on vasculature in LH_{BETA}T_{AG} retinal tumors.

Methods: This study was approved by the Institutional Animal Care and Use Committee and follows Association for Research in Vision and Ophthalmology guidelines. Eighteen-week-old LH_{BETA}T_{AG} retinal tumor eyes (n = 30) were evaluated. Mice were divided into five groups and received periocular injections once weekly for two consecutive weeks of: a) 80% DMSO (dimethyl sulfoxide, vehicle control), b) 0.00333 mg/kg, c) 0.167 mg/kg, d) 3.33 mg/kg, and e) 6.67 mg/kg of rapamycin. Tumor sections were analyzed for hypoxia, tumor burden, and vasculature with immunohistochemistry techniques.

Results: Reduction in tumor burden and hypoxia was significantly different between rapamycin doses and control ($P < 0.002$). Eyes treated with rapamycin at 0.167, 3.33, and 6.67 mg/kg showed a significant decrease in tumor burden in comparison with the vehicle control group ($P = 0.019$, $P = 0.001$, $P = 0.009$, respectively) and the 0.00333 mg/kg dose response ($P = 0.023$, $P = 0.001$, $P = 0.010$, respectively). Eyes treated with rapamycin at 3.33 mg/kg showed a significant reduction in the amount of hypoxia in comparison with the lower concentration groups (0.00333 and 0.167 mg/kg) of rapamycin ($P = 0.024$ and $P = 0.052$, respectively). The number of mature vessels was significantly lower in the 3.33 mg/kg treated versus vehicle control ($P = 0.015$; equal variances assumed, *t*-test for equality of means). The number of neovessels was not significantly different between both groups ($P = 0.092$).

Conclusion: Inhibition of mTOR was shown to reduce tumor burden, hypoxia, and vasculature in the LH_{BETA}T_{AG} retinoblastoma tumor model. Rapamycin may have a role in combination with chemotherapy or other adjuvant therapies to enhance retinoblastoma tumor control.

Keywords: rapamycin, mTOR, hypoxia, retinoblastoma, anaerobic glycolysis

Introduction

Retinoblastoma is the most common primary intraocular malignancy in children.^{1,2} Associated risks of retinoblastoma include metastatic disease, choroidal invasion, and neovascularization.³⁻⁵ More than 95% long-term survival rates in the United States and other developed countries have led to a research focus on local tumor control and globe conservation with preservation of sight. However, present treatments (eg, chemotherapy) result in noteworthy complications including, but not limited to, neutropenia, anemia, thrombocytopenia, infections, and risk for second malignancies (eg, acute myeloid leukemia).⁶ Enucleation is generally performed in about 20% of the cases of intraocular retinoblastoma due to advanced disease.^{3,7}

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The serine–threonine kinase, mammalian target of rapamycin (mTOR), assumes a key regulatory role in cell growth and angiogenesis through effects on cellular metabolism and protein translation. Upstream, mTOR is activated by PI3K/Akt signaling, which has been shown to be dysregulated in cancer cells.^{8,9} As a result, enhanced mTOR activity leads to altered cellular signaling, mediated through the downstream targets such as p70S6K. This pathway has recently been shown to be O₂-sensitive. Hypoxia-induced proliferation of adventitial fibroblast was demonstrated to require the activation of mTOR.¹⁰ Other studies have shown the effects of mTOR inhibition resulting in a reduction of numerous downstream targets including glucose transporter (GLUT)-1, vascular endothelial growth factor (VEGF), and hypoxia inducible factor (HIF)-1 α .^{11,12} Notably, cell proliferation and retinoblastoma (Rb) protein suppression concomitantly inhibited the mTOR pathway.¹³

mTOR as an upstream regulator of HIF, a transcription factor that promotes protein production and glycolytic enzymes and transporters involved in glucose uptake under hypoxic conditions, plays a key role in the metabolic shift from oxidative phosphorylation to anaerobic glycolysis.^{11,12,14} Hypoxic retinoblastoma cells survive under low O₂ tension conditions, which are most prevalent during advanced tumor development.¹⁵ These cells have been shown to be resistant to chemotherapy and radiation, which specifically target the rapidly dividing cells.¹⁶ Hypoxic cells, therefore, may not respond to conventional treatments.^{15,17} These cells rely on anaerobic glycolysis for adenosine triphosphate (ATP) production and survival, which is a significantly less efficient method than oxidative phosphorylation in generating energy from glucose. We have previously shown that hypoxic cells can be targeted by inhibiting aspects of cellular metabolism. Using the glycolytic inhibitor 2-deoxy-D-glucose (2-DG), tumor burden was significantly reduced while effectively decreasing the amount of intratumoral hypoxia in LH_{BETA}T_{AG} retinal tumors.^{15,16,18–20}

Novel therapeutic strategies are lacking to effectively control retinoblastoma without the use of systemic chemotherapy, radiation, or enucleation.^{21–23} With mTOR potentially being an O₂-sensitive pathway that cells may utilize to adapt to harsh tumor microenvironments, treatment with the mTOR inhibitor, rapamycin, may provide a viable mode to modulate the killing of the chemo-resistant hypoxic cell population in LH_{BETA}T_{AG} retinal tumors. The purposes of this study are to: 1) analyze the dose response of rapamycin on tumor burden and hypoxia in LH_{BETA}T_{AG} retinal tumors, and 2) study the treatment effect on vasculature in these retinal tumors.

Materials and methods

LH_{BETA}T_{AG} mouse model for retinoblastoma

The study protocol was approved by the University of Miami Institutional Animal Care and Use Review Board Committee. The LH_{BETA}T_{AG} transgenic mouse model used in this study has been characterized previously.²⁴ This animal model develops bilateral multifocal retinal tumors that are stable and grow at a predictable rate (ie, tumor at 4 weeks is undetectable, at 8 weeks is small, at 12 weeks is medium, and at 16 weeks is large).²⁵

Subconjunctival injections of rapamycin

Eighteen-week-old LH_{BETA}T_{AG} retinal tumor, right eyes, (n=30) were treated and evaluated. Mice were divided into five groups and received periocular injections for two consecutive weeks of: a) 80% dimethyl sulfoxide (DMSO, vehicle control), b) 0.00333 mg/kg, c) 0.167 mg/kg, d) 3.33 mg/kg, and e) 6.67 mg/kg. Since rapamycin is lipophilic and, thus, has a poor water solubility, all the dosages of rapamycin were diluted in 80% DMSO. A total volume of 20 μ L was administered in each injection. Treatment of rapamycin was given once a week, starting at 16 weeks of age. Eyes were enucleated at 1 week following the last treatment. To assess hypoxia, mice received 60 mg/kg of pimonidazole via intraperitoneal injection. Mice were euthanized with CO₂ fumes and eyes were enucleated. Tumor sections were analyzed for hypoxia, tumor burden, and vasculature.

Tumor burden measurements

Eyes were sectioned serially and processed for standard hematoxylin-eosin (H&E) staining. Microscopic images of H&E-stained sections (50 8- μ m sections per eye) were obtained with a digital camera at a magnification of 40 \times . The section of the eye containing the largest cross-sectional tumor area was chosen for analysis. Tumor boundaries were traced using imaging software (Image Pro Express Software; Media Cybernetics, Silver Spring, MD). Tumor areas for all eyes were averaged, yielding an average area for each group. Tumor burden was averaged, yielding an average area for each group. Tumor burden was expressed as the tumor/globe ratio by dividing the tumor area by the area of the globe to normalize the data as previously described.²⁶

Measuring hypoxic regions

To assess tumor hypoxia after treatment, LH_{BETA}T_{AG} mice were injected intraperitoneally with a 0.16 mL suspension of pimonidazole (a drug used to detect hypoxia that penetrates all tissues, including the brain). This suspension consisted

of 10 mg of pimonidazole hydrochloride (Chemicon, Temecula, CA) in 1 mL saline. Pimonidazole is known to bind to thiol-containing proteins in cells under low oxygen (O_2) tension.²⁷ These adducts can be detected with specific antibodies and stained using immunohistochemical techniques. Animals were euthanized 2 hours after pimonidazole injection, and eyes were harvested and sectioned for histopathologic examination. Eyes were fixed with cold methanol for 10 minutes and immunostained with a directly labeled antibody recognizing pimonidazole adducts (Hypoxyprobe 1-Mab-1-FITC, clone 4.3.11.3; Chemicon) or the same concentration of a directly labeled isotype control antibody (mouse IgG1-FITC; Caltag, Burlingame, CA). Background signal intensities were minimal. All samples were normalized to intensities from isotype controls.

Immunohistochemistry

Tumor samples were frozen in OCT (optimal cutting temperature) compound immediately after enucleation, and serially sectioned (8 μ m). Slides were fixed with methanol for 10 minutes (-20°C) and immunohistochemical analyses were performed. Mature vessels were detected with alpha-smooth muscle actin (α -sma) Cy3 conjugate (1:3,000; Sigma Chemical Co, St Louis, MO) which specifically binds to pericytes.²⁸ Neovessels were detected with anti-endoglin (CD105 Wi, 1:500; Abcam, Cambridge, MA), which has been shown to have specificity for endothelial cells undergoing angiogenesis.²⁹ Alexa Fluor 568 goat anti-mouse and 488 donkey anti-mouse were used as secondary antibodies for anti-collagen type IV and endoglin, respectively (1:500; Invitrogen, Carlsbad, CA). Omission of the primary antibody (secondary only) was used as a negative control for nonspecific binding. Cell nuclei were stained for 5 minutes with 4',6'-diamidino-2-phenylindole (DAPI, 1:5,000; Invitrogen, Carlsbad, CA).

Image analysis

Serial cross-sections of eyes containing tumors were examined for the presence of the described markers with a BX51 Olympus upright fluorescence microscope (Olympus American Inc., Melville, NY). All images were obtained at 200 \times magnification using different filters for the DAPI, Alexa Fluor 488, and 568 signals.

Statistical methods

Analysis of variance followed by post hoc least-significant different tests was used to evaluate differences between treatment groups with respect to tumor burden and hypoxia. Differences in the number of new vessels and mature vessels

between the vehicle control and the rapamycin treated group were evaluated by two sample *t*-test. Values were considered significant with *P*-values ≤ 0.05 .

Results

Tumor growth is directly associated with advancing age in the LH_{BETA}T_{AG} transgenic mouse model.³⁰ We have previously shown that hypoxia is significantly detected in large-size LH_{BETA}T_{AG} retinal tumors, and minimal hypoxia is observed in small LH_{BETA}T_{AG} retinal tumors.¹⁵

To assess the impact of periocular administration of rapamycin on tumor burden and hypoxia, LH_{BETA}T_{AG} mice were treated with varying dosages of this mTOR inhibitor. There was no apparent toxicity observed due to the drug at the doses used in the current study. There were highly significant differences between treatment doses for tumor burden and hypoxia ($P < 0.001$). Tumor burden was found to be significantly different between rapamycin doses ($P < 0.002$) (Figures 1 and 2). Eyes treated with rapamycin at 0.167, 3.33, and 6.67 mg/kg showed a significant decrease in tumor burden in comparison with the vehicle control group ($P = 0.019$, $P = 0.001$, $P = 0.009$, respectively) and the 0.00333 mg/kg dose response ($P = 0.023$, $P = 0.001$, $P = 0.010$, respectively). There was no difference between the lowest dose group (ie, 0.00333 mg/kg) and the vehicle control for tumor burden ($P = 0.992$).

The percentage of hypoxia is significantly different between the rapamycin doses ($P < 0.002$) (Figures 2 and 3). Eyes treated with rapamycin at 3.33 mg/kg showed a significant decrease in the percentage of hypoxia in comparison with the

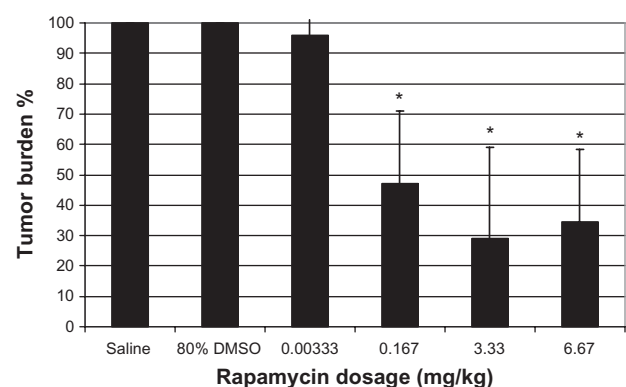


Figure 1 Percentage of tumor burden following different doses of treatment with rapamycin alone. Tumor burden is significantly different between rapamycin doses ($P < 0.002$). Eyes treated with rapamycin at 0.167, 3.33, and 6.67 mg/kg showed a significant decrease in tumor burden in comparison with the vehicle control group ($P = 0.019$, $P = 0.001$, $P = 0.009$, respectively) and the 0.00333 mg/kg dose response ($P = 0.023$, $P = 0.001$, $P = 0.010$, respectively).

Note: *Denotes statistical significant percentage reduction from the vehicle control (80% DMSO).

Abbreviation: DMSO, dimethyl sulfoxide.

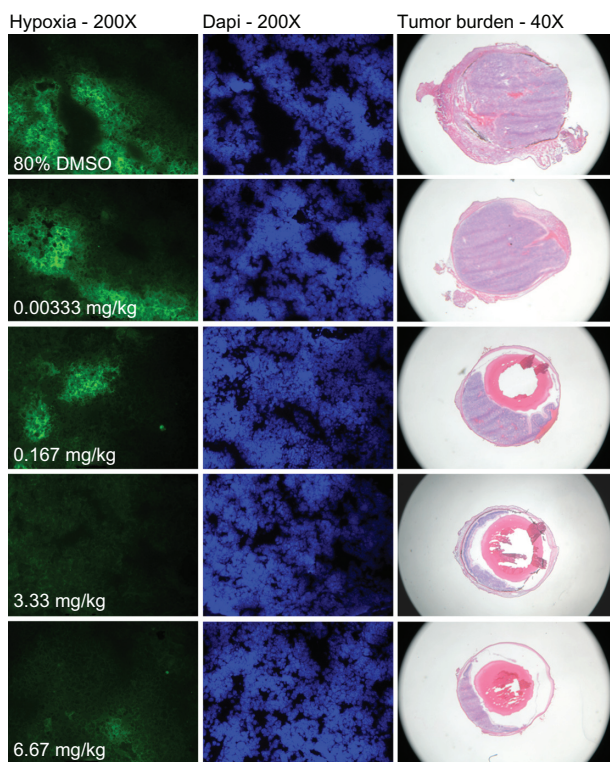


Figure 2 Hypoxia and tumor burden reduction following different doses of rapamycin treatment. There were highly significant interactions between the treatment dose for tumor burden and hypoxia ($P < 0.001$). DAPI stain (blue) for all the cell nuclei and pimonidazole stain (green) for hypoxic regions.
Abbreviations: DAPI, 4',6'-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.

lower concentration groups (0.00333 and 0.167 mg/kg) of rapamycin ($P = 0.024$ and $P = 0.052$, respectively). There was no difference between the highest doses of rapamycin (3.33 and 6.67 mg/kg) for the percentage of intratumoral hypoxia ($P = 0.997$). There is a significant correlation between tumor burden and the percentage of hypoxia ($P < 0.001$; Spearman non-parametric correlation, $r = 0.725$).

To assess the impact of periocular administration of rapamycin on blood vessels, the amount of neovessels and mature vessels were analyzed for the rapamycin dose with the highest impact on the reduction of tumor burden and hypoxia (ie, 3.33 mg/kg). Blood vessels were broken down by vessel caliber (ie, small and large).³¹ The percentage of mature vessels was significantly lower in the rapamycin treated versus the vehicle control group ($P = 0.015$; equal variances assumed, t -test for equality of means) (Figures 4 and 5). The percentage of neovessels was not significantly different between the rapamycin treated and the vehicle control group ($P = 0.092$). This change was mainly due to the small-caliber blood vessels with a reduction of 41.1% for mature vessels versus 70.5% for neovessels compared with the vehicle control ($P < 0.001$). There were no significant changes for large-caliber vessels

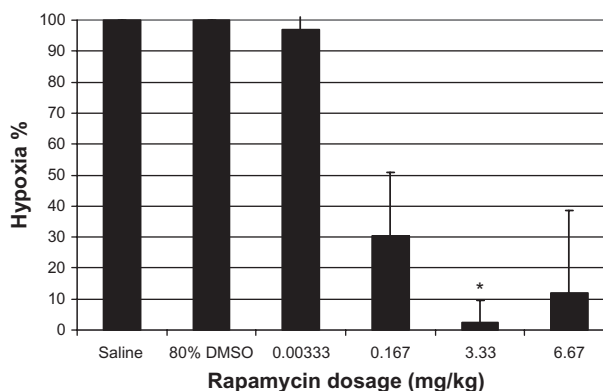


Figure 3 Reduction in the percentage of tumor hypoxia following different doses of treatment with rapamycin. The percentage of hypoxia is significantly different between the rapamycin doses ($P < 0.002$). Eyes treated with rapamycin at 3.33 mg/kg showed a significant decrease in the percentage of hypoxia in comparison with the groups treated with 0.00333 and 0.167 mg/kg of rapamycin ($P = 0.024$ and $P = 0.052$, respectively).

Note: *Denotes statistical significant percentage difference in comparison with the groups treated with 0.00333 and 0.167 mg/kg.
Abbreviation: DMSO, dimethyl sulfoxide.

for neither neo- nor mature vessels compared with the vehicle control (101% and 54.7%, respectively).

Discussion

This study is the first to demonstrate that inhibition of mTOR significantly reduces hypoxia and tumor burden in LH_{BETA} T_{AG} retinal tumors. Tumor cells thrive in a heterogeneous microenvironment that contains regions with low O₂ tensions requiring neoplastic cells to adapt to hypoxic conditions as the tumor develops.^{15,17,32-34} In order to survive under hypoxia, metabolically active tumor cells alter their protein synthesis favoring enhanced anaerobic glucose metabolism. Additional stress

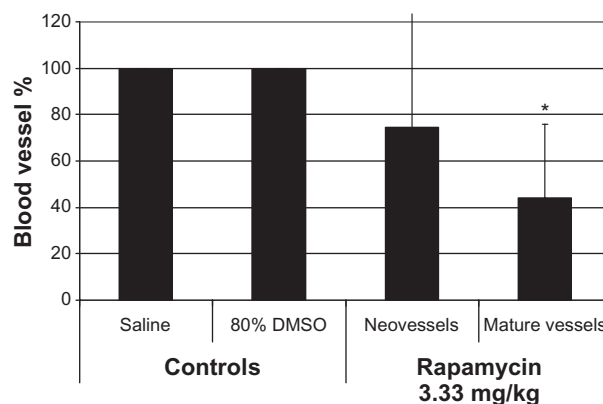


Figure 4 Effect of rapamycin on the vasculature. Neovessels and mature vessels were analyzed for the rapamycin dose with the highest impact on reducing tumor burden and hypoxia (ie, 3.33 mg/kg). The percentage of mature vessels was significantly lower in the rapamycin treated versus the untreated group ($P = 0.015$), whereas the percentage of neovessels was not significantly different between the rapamycin treated and the control group ($P = 0.092$).

Note: *Denotes statistical significant percentage reduction from the vehicle control (80% DMSO).
Abbreviation: DMSO, dimethyl sulfoxide.

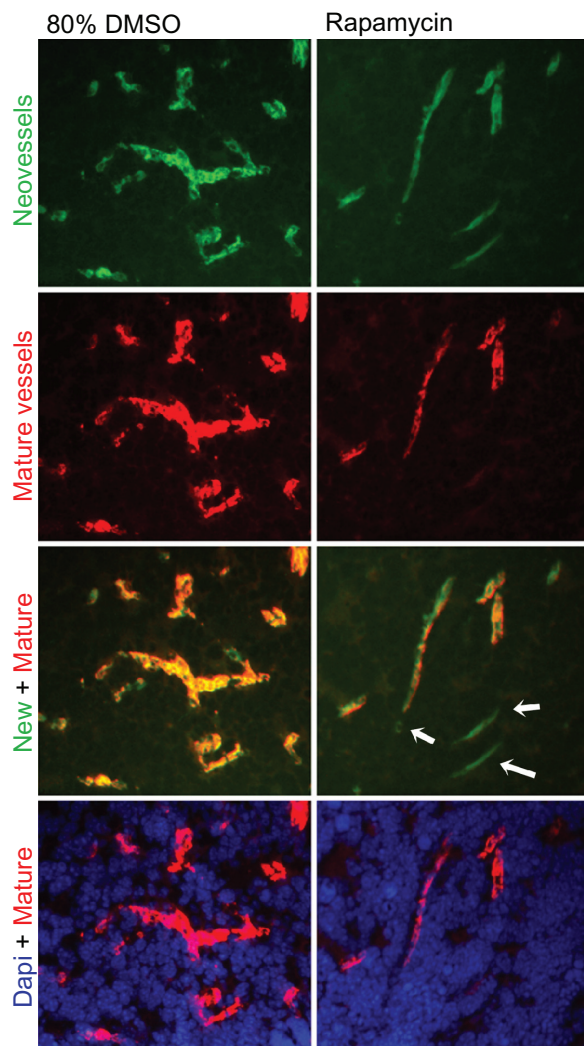


Figure 5 Effect of rapamycin on mature blood vessels. Neovessels and mature vessels were analyzed for the rapamycin dose with the highest impact on reducing tumor burden and hypoxia (ie, 3.33 mg/kg). Although the amount of both neo- and mature vessels decreased following treatment with rapamycin, only the amount of small-caliber, mature vessels decreased significantly ($P = 0.015$). DAPI stain (blue) for all the cell nuclei, endoglin stain (green) for neovessels, and α -sma stain (red) for mature blood vessels. Pictures were obtained at 200 \times high power field.

Note: *Arrows indicate neovessels that do not overlap with mature vessels.

Abbreviations: DAPI, 4',6'-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.

adaptations include mTOR activation, which stimulates HIF-1 through downstream effectors,³⁵⁻³⁷ leading to altered cellular metabolism and angiogenesis.³⁸ As a result, hypoxic cells utilize anaerobic glycolysis for ATP production and rely less on oxidative phosphorylation. With this shift to anaerobic glycolysis, cells selectively alter gene expression to increase glucose transporters and glycolytic enzymes such as hexokinase, which catalyzes the phosphorylation of glucose in the first step of glycolysis. Maher et al recently showed that HIF-1 increases the expression of hexokinase.³⁵ Conversely, hypoxic cells with altered siRNAs or mutations that are unable to activate HIF-1, have a corresponding decrease in hexokinase levels.³⁵

The current study supports the hypothesis that hypoxic cells are targeted by blocking mTOR signaling with rapamycin, leading to a corresponding reduction in tumor burden. The reduction in hypoxia observed from different doses of rapamycin treatment suggests that mTOR upregulation is involved in the survival mechanism used by retinoblastoma cells under hypoxic conditions. In the absence of mTOR, the regular process of HIF-1 proteasome-executing degradation continues,³⁹ thus preventing hypoxic cells from having a corresponding increase in the levels of the enzymes and glucose transporters required to survive under anaerobic or low O_2 partial pressure conditions, making hypoxic cells less adept to rely on anaerobic metabolism.³⁵ The current findings further support the essential role the tumor microenvironment, notably hypoxia, plays in advanced tumor progression.

We have previously shown that 2-DG and 2-fluorodeoxy-D-glucose (2-FDG) successfully kill the chemoresistant, hypoxic cell population and decrease tumor burden in the $LH_{BETA}T_{AG}$ transgenic mouse model of retinoblastoma (work in progress for 2-FDG).^{15,17,32} These glycolytic inhibitors can be used in combination with chemotherapy and anti-angiogenic agents to have a synergistic effect on tumor burden.³² Since mTOR and HIF-1 upregulate the production of glycolytic enzymes, higher amounts of the competitive inhibitor, 2-DG, are essential to inhibit glycolysis. In fact, increased sensitivity of hypoxic cells to 2-DG is correlated to the expression of HIF in different tumor cell lines.⁴⁰ It follows that mTOR inhibition should decrease this resistance to glycolytic inhibition found in tumor cells under hypoxia. Thus, blocking mTOR with rapamycin should not only interfere with the downstream upregulation of anaerobic metabolism but also cause hypoxic cells to become more susceptible to 2-DG. Results from the current study further demonstrate that mTOR may play an essential role in either the cellular shift to anaerobic glycolysis or the anaerobic uptake of glucose. The dose effect response curve obtained for tumor burden and hypoxia following rapamycin treatment provides baseline data to consider combination therapies of mTOR and chemotherapy for vasculature targeting to treat retinoblastoma.

Angiogenesis has been highly correlated with tumor proliferation and metastasis in a number of tumors.⁴¹⁻⁴⁵ Protein synthesis for cell growth, proliferation, and angiogenesis is regulated by mTOR, which controls different signals from growth factor receptors to secure the cell with sufficient nutrients and energy for cell growth. Cancer cells have been shown to have a dysregulation in the angiogenic pathways mediated by mTOR.⁴⁶ Using the $LH_{BETA}T_{AG}$ mouse model of retinoblastoma, we have shown that anti-angiogenic therapy primarily targets

areas with high angiogenic activity, while having little to no effect in established mature blood vessels.³⁰ We have also shown that the heterogeneity and spatial distribution of neovessels and mature vessels in ocular tumors may impact the efficacy of anti-angiogenic therapies, and may dictate the treatment modalities used.^{31,47} Other studies have shown that inhibition of mTOR can potentially inhibit angiogenesis by reducing VEGF-receptors (VEGFRs).^{48,49} VEGFR regulates angiogenic signaling in both endothelial cells and vascular pericytes, mediating tumor proliferation.^{49,50} In the current study, the number of mature blood vessels significantly decreased following treatment with rapamycin, whereas the number of neovessels remained stable following treatment. These results suggest that rapamycin affected pericytes, having little or no effect on endothelial cells. This drug may have had an indirect effect on vascular pericytes by mediating the angiogenic signaling from VEGFR in the tumor microenvironment. In addition, the use of DMSO as the vehicle control may cause nonapparent toxicities on the tumors, affecting the overall effects of angiogenesis.^{51–53} Since anti-angiogenic therapy (ie, anecortave acetate and combretastatin) has no effect in the established, pericyte protected mature blood vessels that are present in advanced LH_{BETA}T_{AG} retinal tumors,³⁰ rapamycin may be an alternative treatment modulator to enhance the effects of anti-angiogenic agents and, thus, their modulation as adjuvant therapies in the treatment of retinoblastoma.^{17,26,30}

In conclusion, we have shown that the mTOR inhibitor rapamycin led to greater tumor control and decreased the amount of hypoxic regions in the LH_{BETA}T_{AG} mouse model for retinoblastoma. The use of rapamycin as an mTOR inhibitor in a preclinical trial has several benefits. Rapamycin is commercially available, has been extensively studied in human clinical trials, and has a unique preparation (topical delivery).^{54–58} We believe that mTOR is a potential target in retinoblastoma and its modulation may allow a synergistic impact on tumor burden control in combination with standard treatment modalities (eg, chemotherapy) and other adjuvant therapies (eg, glycolytic inhibitors and anti-angiogenic agents) to treat retinoblastoma tumors. Future studies should include testing the compatibility of different treatment dose and schedule combinations for optimal retinoblastoma tumor burden control.

Notes

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Disclosure

The authors report no conflicts of interest in this work.

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