Retinoblastoma Treatment: Utilization of the Glycolytic Inhibitor, 2-deoxy-2-fluoro-D-glucose (2-FG), to Target the Chemoresistant Hypoxic Regions in LH_{BETA}T_{AG} Retinal Tumors

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PURPOSE. To analyze the effect of the glycolytic inhibitor 2-deoxy-2-fluoro-D-glucose (2-FG) on tumor burden, hypoxia, and blood vessels in LH_{BETA}T_{AG} retinal tumors.

METHODS. Seventeen-week-old LH_{BETA}T_{AG} retinal tumor eyes ($n = 36$) were treated with 2-FG and analyzed at 1 day and 1 week post a single treatment, and 1 day post a biweekly treatment for 3 weeks. Tumor sections were analyzed for hypoxia, tumor burden, and vasculature. To assess tumor burden, sections were processed for standard hematoxylin-eosin (H&E) staining. Immunofluorescent techniques were used to stain for new and mature blood vessels.

RESULTS. Hypoxia and tumor burden reduction are significantly different between the treatment schedules used ($P < 0.001$). Eyes treated with 2-FG for 3 weeks showed a significant decrease in hypoxia ($P = 0.001$) and tumor burden ($P = 0.009$); whereas those treated with one injection and evaluated at 1 day and 1 week postinjection did not show a decrease in either hypoxia ($P = 0.373$ and $P = 0.782$, respectively) or tumor burden ($P = 0.203$ and $P = 0.836$, respectively). When evaluating the spatial distribution of hypoxic regions in the different areas of the tumor, 2-FG showed a differential effect on hypoxia depending on the area. Hypoxia was most decreased in the base of the treated eyes with a 95% reduction ($P < 0.001$).

CONCLUSIONS. This is the first study to elucidate that 2-FG treatment in retinoblastoma produces an impact on hypoxia and a concomitant decrease on tumor burden. In this study, the authors validate their previous studies by revealing that glycolytic inhibitors effectively target hypoxia in retinoblastoma tumors. The future application of 2-FG as an adjuvant treatment to standard chemotherapy may enhance the treatment of retinoblastoma. (*Invest Ophthalmol Vis Sci.* 2012;53:996–1002) DOI:10.1167/iovs.11-8265

Retinoblastoma, as the most frequent primary intraocular malignancy in children,^{1,2} accounts for 4% of all the pediatric malignancies in the United States.³ Survival rates now approach greater than 95%, partially due to major advances in treatment in pediatric oncology and research focus on tumor control.⁴ However, 80% of all the pediatric malignancies occur in developing countries, where treatment is limited and long-term survival rates are low. In addition, existing therapies for retinoblastoma (i.e., chemotherapy and focal therapies) result in unfavorable outcomes at both the systemic and local levels.^{4–6}

The retinoblastoma tumor presents with a heterogeneous microenvironment comprised of regions with high angiogenic activity and other areas with low oxygen (O_2) tension conditions, which become more pronounced during advanced disease.⁵ Tumor cells that survive under these hypoxic regions have been shown to be resistant to chemotherapy and radiation.⁶ These standard treatment modalities selectively target the rapidly dividing cell populations. Cells under hypoxia rely on anaerobic glycolysis for ATP production, a less resourceful means than oxidative phosphorylation for glucose catabolism, and their cellular metabolism slows down significantly for energy preservation. Therefore, these cells may not respond to conventional treatments such as chemotherapy, which mainly target the rapidly proliferating cells.^{5,7} In previous studies, we demonstrated that hypoxic cells can be killed in the LH_{BETA}T_{AG} retinal tumor by using the glycolytic inhibitor 2-deoxy-2-fluoro-D-glucose (2-DG). In these studies, tumor burden was significantly reduced while effectively decreasing the amount of intratumoral hypoxia.^{5,6,8–10} 2-DG was also shown to impact angiogenesis both in vitro and in vivo.¹¹

The glycolytic inhibitor 2-deoxy-2-fluoro-D-glucose (2-FG) has been shown to be a more potent glycolytic inhibitor than 2-DG under low O_2 tension conditions in vitro.⁹ Glycolysis inhibition as measured by lactate levels and cytotoxicity, was found to be more pronounced using 2-FG than 2-DG in osteosarcoma (143B) growing anaerobically by treatment with the ATP synthase inhibitor oligomycin and knockout mtDNA mutant cells.⁹ Because standard treatment for retinoblastoma is related to a high morbidity and potential mortality, novel and adjunct therapies are needed to treat this devastating disease.^{12–14} We hypothesize that 2-FG effectively targets the intratumoral cells under hypoxic conditions in vivo using our LH_{BETA}T_{AG} animal model. Therefore, the purpose of this study is to analyze the effect of the glycolytic inhibitor 2-FG on tumor burden, hypoxia, and blood vessels in LH_{BETA}T_{AG} retinal tumors.

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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, and all animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The LH_{BETA}T_{AG} transgenic mouse model used in this study has been characterized previously.¹⁵ This animal model develops bilateral multifocal retinal tumors that are stable and grow at a predictable rate (i.e., tumor at 4 weeks is undetectable, at 8 weeks is small, at 12 weeks is medium, and at 16 weeks is large).¹⁶

Subconjunctival Injections of 2-FG

Seventeen-week-old LH_{BETA}T_{AG} retinal tumor right eyes ($n = 36$) were treated and evaluated. Mice were divided into three equal groups and received periocular injections of either 2-FG (100 mg/mL; Sigma Chemical Co., St. Louis, MO) or balanced salt solution (vehicle control; BSS; Alcon Laboratories, Inc., Fort Worth, TX) for: one time and animals were euthanized 1 day posttreatment (1 d Tx); one time and animals were euthanized 1 week posttreatment (1 wk Tx); and twice a week for three weeks and animals were euthanized 1 day after the last treatment (3 wk Tx). A total volume of 20 μ L was administered in each injection. 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.

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 O₂ tension.¹⁷ 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.

Both the density and the spatial distribution of hypoxic regions were analyzed independently. The density of hypoxia was measured by calculating the ratio of the amount of pixels stained with pimonidazole (i.e., marker for hypoxia) over the amount of pixels for total tumor area (100 $\times$ HPF; Adobe Photoshop CS5; Adobe, San Jose, CA). The spatial distribution of hypoxia in each tumor was performed by doing a grading analysis of different size hypoxic areas (i.e., small, medium, large, and very large) present in each intratumoral region (i.e., apex, center, lateral, and base; 100 $\times$ HPF). The different size hypoxic areas were labeled based on a cell count in the following manner: small size hypoxic areas were represented by ≤ 20 hypoxic cells and were assigned a value of 1; medium size hypoxic areas were represented by 21 to 40 hypoxic cells and were assigned a value of 2; large size hypoxic areas were represented by 41 to 80 hypoxic cells and were assigned a value of 4; and very large size hypoxic areas were represented by ≥ 81 hypoxic cells and were assigned a value of 8. A final value for each intratumoral region (e.g., apex) in all the tumors was obtained by adding all the values assigned for the different size hypoxic areas.

Tumor Burden Measurements

Eyes were sectioned serially and processed for standard hematoxylin-eosin (H&E) staining. Microscopic images of H&E-stained sections (8

μ m sections per eye) were obtained with a digital camera at a magnification $\times 40$. 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.¹⁸

Immunohistochemistry

Tumor samples were frozen in OCT 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 α -smooth muscle actin (α -sma) Cy3 conjugate (1:3000; Sigma Chemical Co.) which specifically binds to pericytes.¹⁹ 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.²⁰ Alexa Fluor 488 donkey anti-mouse was used as a secondary antibody for endoglin (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, 1:5000; Invitrogen). Blood vessel caliber analysis and grading was performed as previously described.²¹

Image Analysis

Serial cross-sections of eyes containing tumors were examined for the presence of the described markers with an upright fluorescence microscope (BX51 Olympus; Olympus American Inc., Melville, NY). All images were obtained at magnification $\times 100$ and $\times 200$ using different filters for the DAPI, Alexa Fluor 488, and 568 signals.

Statistical Methods

Analysis of variance followed by two-sample *t*-test was used to evaluate differences between treatment groups with respect to hypoxia and tumor burden. Results are reported from untransformed data with square root *P* values. The differences between treatments by location in the spatial distribution of hypoxia were made using a Greenhouse-Geisser corrected repeated measures analysis of variance. Differences in the percent reduction of new vessels and mature vessels after 2-FG treatment from the vehicle control and were evaluated by one- and two-sample *t*-test. Values were considered significant with $P \leq 0.05$.

RESULTS

We have previously shown that tumor growth is directly associated with advancing age in the LH_{BETA}T_{AG} transgenic mouse model.²² Additionally, we reported that large regions of hypoxia are present in advanced disease in the retinoblastoma animal model, whereas small regions of hypoxia are present in these tumors during early stages of the disease.⁵ In the present study, LH_{BETA}T_{AG} transgenic mice were treated with periocular injections of 2-FG following three treatment schedules: one injection and tumors were analyzed 1 d Tx; one injection and tumors were analyzed 1 wk Tx; and two injections per week for three weeks and tumors were analyzed 3 wk Tx. There was no apparent significant toxicity observed due to the drug at the dose used in the present study.

2-FG Treatment Decreases Hypoxia in Retinoblastoma

Hypoxia is significantly different between the treatment schedules used ($P < 0.001$; Figs. 1–4). Hypoxia is decreased after 2-FG treatment compared with controls (Figs. 1, 2).

FIGURE 1. Intratumoral hypoxia after treatment with one injection of 2-FG. Hypoxia is significantly different between the treatment schedules used ($P < 0.001$), even though the difference between one injection (inj) treatment and control is nonsignificant for both 1 day (1 d) and 1 week (1 wk) postinjection ($P = 0.373$ and $P = 0.782$, respectively). However, hypoxia is decreased substantially at 1 d post treatment after one injection.

Eyes treated with one injection of 2-FG and analyzed at 1 d and 1 wk did not show a statistically significant difference between the treated group and the control ($P = 0.373$ and $P = 0.782$, respectively), while eyes treated with six injections of 2-FG for 3 weeks showed a significant decrease in hypoxic density ($P = 0.001$; Table 1; Figs. 3, 4).

Because we had previously observed differences in hypoxia according to the location within retinoblastoma (i.e., apex, center, lateral, base), the regional effects of 2-FG on hypoxia were evaluated. The difference among all treatment groups for hypoxia was highly significant ($P < 0.001$; Fig. 5). In addition, there is a statistically significant interaction in the differences between treatments by location ($P < 0.008$; Greenhouse-Geisser corrected repeated measures analysis of variance; Table 2). Our results suggest that 2-FG has a differential effect on hypoxia depending on the intratumoral region analyzed. Specifically, hypoxia was decreased in the center and lateral areas of the treated eyes by 81% and 79%. Further, hypoxia was reduced in the apex and the base of the treated eyes by 88% and 95% (Fig. 5).

FIGURE 3. Reduction of tumor hypoxia after treatment with six injections of 2-FG for 3 weeks. Hypoxia is significantly different between the one injection treatment schedules used (1 d and 1 wk) and the six injections treatment schedule (1 wk; $P < 0.001$). Eyes treated with 2-FG for 3 weeks showed a significant decrease in hypoxic density (* $P = 0.001$).

2-FG Causes a Concomitant Reduction in Tumor Burden in Retinoblastoma

Tumor burden is significantly different between the treatment schedules used ($P < 0.001$; Figs. 6, 7). Eyes treated with 2-FG for 3 weeks showed a significant decrease in tumor burden ($P = 0.009$); whereas those treated for 1 d and 1 wk did not ($P = 0.203$ and $P = 0.836$, respectively; Table 1).

Impact on Vasculature after Treatment with 2-FG

The amount of blood vessel reduction after treatment with 2-FG was analyzed by doing a vessel count of small-, medium-, and large-caliber vessels. Analysis was done using mean percent differences from 100% controls. The total blood vessel percent decrease was statistically significant for both new and mature blood vessels ($P = 0.025$ and $P < 0.001$, respectively) associated with the use of 2-FG, with a 29% reduction of new

FIGURE 2. Hypoxia density after two treatment schedules with one injection of 2-FG. There were highly significant interactions between the control groups (A, B, and E, F) and the 2-FG treated groups (C, D, and G, H; $P < 0.001$). Hypoxia is decreased after treatment with 2-FG for 1 d, although not significantly (C, D; $P = 0.001$). Blue, DAPI stain for all the cell nuclei; green, pimonidazole stain for hypoxic regions. Pictures were obtained at magnification $\times 100$ high power field.

and 62% reduction of mature blood vessels from nontreated groups. This impact can be seen as early as 1 wk post treatment ($P = 0.029$) and continues through the entire 3 week treatment ($P = 0.001$). The total blood vessel percent reduction for all blood vessel calibers associated with 2-FG treatment was statistically significant regardless of the vessel caliber analyzed ($P = 0.05$ for small-caliber [28%], $P = 0.008$ for medium-caliber [37%], and $P = 0.05$ for large-caliber [49%]; Fig. 8).

DISCUSSION

During tumorigenesis, the highly metabolic tumor cells require a steady supply of O₂ and nutrients to thrive and proliferate, which is usually associated with angiogenesis and regulatory growth factors. During later stages of tumor growth, a discrepancy of O₂ metabolism leads to concentrated areas with lower O₂ partial pressures and a disorderly microvasculature, which further produces a microenvironment depleted of O₂ and blood flow.^{23–25} Under these harsh conditions, tumor cells experience a switch in their metabolic machinery to survive by relying on glucose consumption and anaerobic glycolysis, which is a less efficient method of ATP production than oxidative phosphorylation.²⁶ To conserve energy, the hypoxic cells proliferate at a much slower rate than the typical abnor-

mal tumor cells; therefore, becoming resistant to antiangiogenic, chemotherapeutic, and radiation therapy treatments that target the rapidly dividing cells.²⁷

Because these cells become heavily dependent on glucose consumption under anaerobic conditions, therapeutic agents that obstruct glycolysis have been explored. It has been shown that the glycolytic inhibitor 2-DG effectively targets these hypoxic cells in retinoblastoma tumors in vivo^{5,10} and in osteosarcoma (143B) and knockout mtDNA mutant cells in vitro.⁹ Moreover, a combination therapy of 2-DG and chemotherapy (i.e., carboplatin), significantly reduced tumor burden in LH_{BETA}T_{AG} retinal tumors more efficiently than when either treatment was provided alone.⁵ Because the commercially available 2-FG was demonstrated to be a more effective glycolytic inhibitor in vitro than 2-DG in targeting hypoxic cells,⁹ in the present study we tested the efficacy of focal, periocular delivery of 2-FG in vivo in the LH_{BETA}T_{AG} transgenic mouse model of retinoblastoma.

In the present study, focal delivery of 2-FG decreased hypoxia after 2-FG injections with minimal toxicities associated with the treatment. Results suggest that longer, biweekly treatments (i.e., 2-FG treatment for 3 weeks) in later stages of tumor growth produce a significant reduction on tumor hypoxia and tumor burden at the dosages used. This outcome demonstrates

TABLE 1. Treatment Schedule Effect on Hypoxia and Tumor Burden

Schedule	Group	Hypoxia Density (per 100×HPF)	Hypoxia <i>P</i>	Tumor Burden (mm ²)	Tumor Burden <i>P</i>
		Mean (SD)		Mean (SD)	
1 d (one inj)	Control	1.03 (1.76)	0.373	179844 (77150)	0.203
	2-FG	0.0068 (0.0062)		106210 (43498)	
1 wk (one inj)	Control	1.84 (0.053)	0.782	593238 (489099)	0.836
	2-FG	2.41 (2.63)		677717 (734083)	
3 wk (six inj)	Control	6.42 (2.77)	0.001	669250.84 (0.000022)	0.009
	2-FG	1.92 (1.70)		318185.88 (0.000026)	

Hypoxia and tumor burden are significantly different between the treatment schedules used ($P < 0.001$). Eyes treated with 2-FG for 3 weeks showed a significant decrease in hypoxia and tumor burden ($P = 0.001$ and $P = 0.009$, respectively).

FIGURE 5. Spatial distribution of hypoxia after treatment with 2-FG. The spatial distribution of hypoxia in the different areas of the tumor (i.e., apex, center, lateral, base) was evaluated by counting the amount of small, medium, large, and very large areas of hypoxia in each of the intratumoral regions (i.e., apex, center, lateral, and base; 100 × HPF). Grading scale measuring the size of the hypoxic areas and the amount of these areas per intratumoral region: Small size hypoxic areas were represented by ≤20 hypoxic cells and were assigned a value of 1; medium size hypoxic areas were represented by 21 to 40 hypoxic cells and were assigned a value of 2; large size hypoxic areas were represented by 41 to 80 hypoxic cells and were assigned a value of 4; and very large size hypoxic areas were represented by ≥81 hypoxic cells and were assigned a value of 8. The difference between treated and untreated was highly significant (* $P < 0.001$) and there is a statistically significant interaction in the differences between treatments by location ($P < 0.008$). 2-FG has a differential effect on hypoxia depending on the intratumoral area: apex (88%), center (81%), lateral (79%), and base (95%).

that 2-FG is a potent glycolytic inhibitor in targeting hypoxic cells in retinoblastoma and further corroborates our previous results showing that glycolytic inhibitors can be used as adjuvant to standard treatment modalities (e.g., chemotherapy) to target the chemoresistant, hypoxic cells. Moreover, histopathological examinations of H&E staining of the LH_{BETA}T_{AG} retinal tumor sections did not display any toxicity or abnormality in the eye associated with drug delivery, further revealing that periocular delivery of this drug is a viable and efficient treatment modality.

Furthermore, we analyzed the effects of 2-FG in the different areas of the tumor. As previously characterized, the basal and central regions of retinal tumors display the highest amount of hypoxia, whereas the other regions (i.e., apex, lateral) show little to no hypoxia.⁵ This study is the first to show that 2-FG decreases the amount of intratumoral hypoxia in vivo in all the different areas analyzed. Our results showing that the basal regions had the greatest percentage of reduced hypoxia may be explained by the findings that control eyes present with a much higher density of hypoxic cells in these

FIGURE 6. Reduction of tumor burden after treatment with 2-FG. Tumor burden is significantly different between the treatment schedules used (* $P < 0.001$). Eyes treated with 2-FG for 3 weeks showed a significant decrease in tumor burden ($P = 0.009$); whereas those treated for 1 d and 1 wk did not show a decrease in hypoxia ($P = 0.203$ and $P = 0.836$, respectively).

areas. Further, basal regions are potentially exposed to higher drug concentrations.

A proposed mechanism of 2-FG in effectively eliminating hypoxic cells is that it interferes with glycolysis through competitively inhibiting phosphoglucose isomerase (PGI) and allosterically inhibiting hexokinase. It is hypothesized that because tumor cells have to sustain high demands of ATP necessary for their development, they express higher levels of the hexokinase but not PGI.^{28,29} Glycolysis inhibition seems to be a promising tool to target the hypoxic cells that are associated with chemotherapy and radiation therapy resistivity, by interfering with the glycolytic pathway through inhibition of these enzymes.⁵ Data indicate that the competitive inhibition of PGI occurs at a lower K_m for 2-DG and perhaps 2-FG than that of hexokinase. Thus, there appears to be a hierarchy where either of these glycolytic inhibitors primarily affect PGI and only at significantly higher concentrations affect hexokinase. If indeed hexokinase is shut down then glucose-6-phosphate levels required for the functioning of the pentose phosphate shunt will be too low. If the pentose shunt is blocked effects on fatty acid synthesis and glutathione function would be expected to be detrimental to the cell.

In addition, it has also been reported that angiogenesis, the development of new blood vessels from pre-existing ones, is also affected with the glycolytic inhibitor 2-DG in the LH_{BETA}T_{AG} transgenic mouse model.³⁰ We have previously reported that the mammalian target of rapamycin (mTOR), which is an upstream regulator of glycolysis, targeted mature blood vessels in the same animal model.³¹ These facts suggest that drugs implicated in glucose catabolism have an effect on angiogenesis and/or blood vessel maturation. In the present study, treatment with 2-FG effectively decreased both the percentage of new, mature, and total blood vessels in the retinal tumors of the transgenic animal model of retinoblastoma. Previous reports in

TABLE 2. The Spatial Differential Distribution of Hypoxia through the Different Regions of the Tumor after 2-FG Treatment for 3 Weeks

	Apex	Center	Lateral	Base
Control	3.22 (3.49)	8.14 (4.87)	2.92 (3.57)	9.72 (4.74)
2-FG (six inj)	0.375 (0.744)*	1.56 (1.80)*	0.625 (0.954)*	0.563 (1.24)*

The spatial distribution of hypoxia (mean amount of hypoxia per 100 × HPF) in the different areas of the tumor (i.e., apex, center, lateral, base) was evaluated. Results suggest that 2-FG has a differential effect on hypoxia depending on the intratumoral area.

* The difference between treated and untreated was highly significant ($P < 0.001$) and there is a statistically significant interaction in the differences between treatments by location ($P < 0.008$; Greenhouse-Geisser corrected repeated measures analysis of variance).

FIGURE 7. Tumor burden reduction after treatment with 2-FG. Representative H&E pictures of sections (40 × HPF) containing LH_{BETA}T_{AG} retinal tumors that were treated for 1 week (A, B) and 3 weeks (C, D). Only those eyes that were treated with 2-FG for 3 weeks showed a significant decrease in tumor burden ($P = 0.009$).

which 2-DG showed anti-angiogenic activity in vitro in HUVEC cells was revealed to be due to 2-DG's activity as a mannose mimetic interfering with oligosaccharide synthesis resulting in endoplasmic reticulum stress mediated apoptosis. Thus, it appeared that 2-DG's dual activity as inhibitor of glycosylation as well as glycolysis contributed to its overall activity in reducing hypoxic tumor cells. 2-DG's glycolytic inhibitor analog 2-FG was previously shown to be more potent in inhibiting glycolysis than 2-DG and thereby killing hypoxic cells in vitro.⁹ Because in the present study 2-FG targets both new and mature blood vessels, it is possible that 2-FG has a dual action on glycolysis and growth factors associated with blood vessel development, thus, affecting several mechanisms associated with tumor cell development under hypoxic conditions in vitro.

The present results substantiate previous in vitro results that the glycolytic inhibitor 2-FG selectively targets the

chemoresistant, hypoxic cells in LH_{BETA}T_{AG} retinal tumors. Because the effects of 2-FG in the present study mimic those of 2-DG, these results also validate the use of glycolytic inhibitors in the treatment of retinoblastoma. The study additionally provides the rationale for using the retinoblastoma model in the future evaluations of glycolytic inhibitors in combination with other drugs (i.e., chemotherapy and radiation therapy) in several cancers.⁹

In conclusion, our results further demonstrate that hypoxic regions are most pronounced during later stages of the disease that develop in the LH_{BETA}T_{AG} transgenic mice containing retinal tumors. This is the first study to elucidate that periocular administration of 2-FG in this animal model produces an impact on hypoxia and a concomitant influence on tumor burden control. This study correspondingly validates our previous trials revealing that glycolytic inhibitors favor the targeting of the chemoresistant, hypoxic cells in retinoblastoma tumors. The future application of periocular 2-FG as an adjuvant treatment to standard chemotherapeutic agents can be explored to potentially enhance the treatment of pediatric retinoblastoma.

References

1. Pendergrass TW, Davis S. Incidence of retinoblastoma in the United States. *Arch Ophthalmol*. 1980;98:1204-1210.
2. Tamboli A, Podgor MJ, Horm JW. The incidence of retinoblastoma in the United States: 1974 through 1985. *Arch Ophthalmol*. 1990;108:128-132.
3. Abramson DH. Retinoblastoma in the 20th century: past success and future challenges the Weisenfeld lecture. *Invest Ophthalmol Vis Sci*. 2005;46:2683-2691.
4. Laurie NA, Schin-Shih C, Dyer MA. Targeting MDM2 and MDMX in retinoblastoma. *Curr Cancer Drug Targets*. 2007;7:689-695.
5. Boutrid H, Jockovich ME, Murray TG, et al. Targeting hypoxia, a novel treatment for advanced retinoblastoma. *Invest Ophthalmol Vis Sci*. 2008;49:2799-2805.

FIGURE 8. Percent reduction of total blood vessels after treatment with 2-FG. The total blood vessel percent reduction for all blood vessel calibers associated with 2-FG treatment was statistically significant regardless of the vessel-caliber analyzed ($P = 0.05$ for small-caliber [28%], $P = 0.008$ for medium-caliber [37%], and $P = 0.05$ for large-caliber [49%]).

6. Maschek G, Savaraj N, Priebe W, et al. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. *Cancer Res.* 2004;64:31–34.
7. Boutrid H, Pina Y, Cebulla CM, et al. Increased hypoxia following vessel targeting in a murine model of retinoblastoma. *Invest Ophthalmol Vis Sci.* 2009;50:5537–5543.
8. Maher JC, Krishan A, Lampidis TJ. Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-D-glucose in tumor cells treated under hypoxic vs aerobic conditions. *Cancer Chemother Pharmacol.* 2004;53:116–122.
9. Lampidis TJ, Kurtoglu M, Maher JC, et al. Efficacy of 2-halogen substituted D-glucose analogs in blocking glycolysis and killing “hypoxic tumor cells”. *Cancer Chemother Pharmacol.* 2006;58:725–734.
10. Boutrid H, Pina Y, Cebulla C, et al. Vessel targeting increases hypoxia in a murine model of retinoblastoma. *Invest Ophthalmol Vis Sci.* 2009;50:5537–5543.
11. Merchan JR, Kovacs K, Railsback JW, et al. Antiangiogenic activity of 2-deoxy-D-glucose. *PLoS One.* 5:e13699.
12. Berman EL, Donaldson CE, Giblin M, Martin FJ. Outcomes in retinoblastoma. 1974–2005: the Children’s Hospital, Westmead. *Clin Experiment Ophthalmol.* 2007;35:5–12.
13. Rouic LL, Aerts I, Levy-Gabriel C, et al. Conservative treatments of intraocular retinoblastoma. *Ophthalmology.* 2008;115:1405–1410, 1410.e1–2.
14. Scheffer AC, Ciciarelli N, Feuer W, Toledano S, Murray TG. Macular retinoblastoma: evaluation of tumor control, local complications, and visual outcomes for eyes treated with chemotherapy and repetitive foveal laser ablation. *Ophthalmology.* 2007;114:162–169.
15. Windle JJ, Albert DM, O’Brien JM, et al. Retinoblastoma in transgenic mice. *Nature.* 1990;343:665–669.
16. Jockovich ME, Pina Y, Alegret A, Cebulla C, Feuer W, Murray TG. Heterogeneous tumor vasculature in retinoblastoma: implications for vessel targeting therapy. *Retina.* 2008;28:S81–S86.
17. Varia MA, Calkins-Adams DP, Rinker LH, et al. Pimonidazole: a novel hypoxia marker for complementary study of tumor hypoxia and cell proliferation in cervical carcinoma. *Gynecol Oncol.* 1998;71:270–277.
18. Jockovich ME, Murray TG, Escalona-Benz E, Hernandez E, Feuer W. Anecortave acetate as single and adjuvant therapy in the treatment of retinal tumors of LH(BETA)T(AG) mice. *Invest Ophthalmol Vis Sci.* 2006;47:1264–1268.
19. Nehls V, Drenckhahn D. Heterogeneity of microvascular pericytes for smooth muscle type alpha-actin. *J Cell Biol.* 1991;113:147–154.
20. Tanaka F, Otake Y, Yanagihara K, et al. Evaluation of angiogenesis in non-small cell lung cancer: comparison between anti-CD34 antibody and anti-CD105 antibody. *Clin Cancer Res.* 2001;7:3410–3415.
21. Pina Y, Boutrid H, Scheffer A, et al. Blood vessel maturation in retinoblastoma tumors: spatial distribution of neovessels and mature vessels and its impact on ocular treatment. *Invest Ophthalmol Vis Sci.* 2009;50:1020–1024.
22. Jockovich ME, Bajenaru ML, Pina Y, et al. Retinoblastoma tumor vessel maturation impacts efficacy of vessel targeting in the LH-(BETA)T(AG) mouse model. *Invest Ophthalmol Vis Sci.* 2007;48:2476–2482.
23. Konerding MA, Miodonski AJ, Lametschwandtner A. Microvascular corrosion casting in the study of tumor vascularity: a review. *Scanning Microsc.* 1995;9:1233–1243; discussion 1243–1234.
24. Darland DC, D’Amore PA. Blood vessel maturation: vascular development comes of age. *J Clin Invest.* 1999;103:157–158.
25. Gee MS, Procopio WN, Makonnen S, Feldman MD, Yeilding NM, Lee WM. Tumor vessel development and maturation impose limits on the effectiveness of anti-vascular therapy. *Am J Pathol.* 2003;162:183–193.
26. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer.* 2003;3:401–410.
27. Chan HS, Gallie BL, Munier FL, Beck Popovic M. Chemotherapy for retinoblastoma. *Ophthalmol Clin North Am.* 2005;18:55–63, viii.
28. Geschwind JF, Georgiades CS, Ko YH, Pedersen PL. Recently elucidated energy catabolism pathways provide opportunities for novel treatments in hepatocellular carcinoma. *Expert Rev Anticancer Ther.* 2004;4:449–457.
29. Pedersen PL, Mathupala S, Rempel A, Geschwind JF, Ko YH. Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention. *Biochim Biophys Acta.* 2002;1555:14–20.
30. Merchan JR, Kovacs K, Railsback JW, et al. Antiangiogenic activity of 2-deoxy-D-glucose. *PLoS One.* 2010;5:e13699.
31. Pina Y, Decatur C, Murray T, et al. Advanced retinoblastoma treatment: targeting hypoxia by inhibition of the mammalian target of rapamycin (mTOR) in LH(BETA)T(AG) retinal tumors. *Clin Ophthalmol.* 2011;5:337–343.