Evaluation of the *In vitro* and *In vivo* Antitumor Activity of Histone Deacetylase Inhibitors for the Therapy of Retinoblastoma

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**Abstract**

**Purpose:** To evaluate the potential utility of histone deacetylase inhibitors (HDACi) for treatment of retinoblastoma (RB).

**Experimental Design:** Growth-inhibitory effects of HDACi [trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), or MS-275] were assessed in human and transgenic murine RB cells. Effects of TSA and MS-275 were also assessed in combination with standard therapeutic agents for RB. Proapoptotic effects of MS-275 and TSA were evaluated by caspase-3/7 activity, Annexin V translocation, and/or Bim expression analyses. Effects of MS-275 on cell cycle distribution and reactive oxygen species levels were determined by flow cytometry. Retinal tissue morphology was evaluated in mice after local administration of MS-275. Analysis of retinal acetyl-histone levels was used to assess MS-275 delivery after systemic administration. Therapeutic effects of MS-275 were determined in transgenic mouse and rat ocular xenograft models of RB after i.p. injection of 20 mg/kg every other day for 21 or 13 days, respectively.

**Results:** TSA, SAHA, and MS-275 dose dependently reduced RB cell survival. TSA and MS-275 showed additive growth-inhibitory effects in combination with carboplatin, etoposide, or vincristine. TSA and MS-275 increased caspase-3/7 activity. MS-275 increased Annexin V membrane translocation and induced G1 arrest. Cytotoxicity of MS-275 was dependent on increased reactive oxygen species levels and was reversed by antioxidant pretreatment. Intraocular administration of 1 μL of 10 μmol/L MS-275 did not alter ocular tissue morphology. Increased acetyl-histone levels confirmed MS-275 delivery to retinal tissue after systemic administration. MS-275 significantly reduced tumor burden in both mouse and rat models of RB.

**Conclusions:** HDACi should be considered for clinical trials in children with RB.

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Cancer is a genetic disease. Tumorigenesis arises from a stepwise accumulation of DNA alterations resulting in gain of function of oncogenes or loss of function of tumor suppressor genes (1). Epigenetic changes, such as DNA methylation and histone acetylation/deacetylation, also contribute to tumor development by regulating the transcriptional activity of genes, including those important for cellular proliferation, differentiation, and survival (2–4). Histone acetylases and histone deacetylases (HDACs) regulate transcription through the transfer and removal of acetyl groups on lysine residues at the NH2 terminus of histones, the major protein component of chromatin (5). In general, acetylated histones force chromatin into an open conformational state that promotes gene transcription, whereas deacetylated histones maintain chromatin in a condensed state that silences transcription. Histone acetylases and HDACs target many other proteins in addition to histones (6), and deregulation of these interactions can also be tumorgenic (7, 8). Nonhistone targets of histone acetylases and HDACs include numerous proteins implicated in tumor initiation or progression, including p53, c-MYC, nuclear factor-xB, and E2F.

Retinoblastoma (RB) affects 1 in 15,000 children and accounts for 12% of infant cancers (9, 10). Standard treatment options for this disease are limited and associated with significant toxicities. First-line therapy at most centers includes combination chemotherapy with adjuvant focal therapy (laser therapy, cryotherapy, or brachytherapy). The standard regimen is carboplatin and etoposide or teniposide, with or without vincristine (11). Carboplatin is associated with nephrotoxicity as well as ototoxicity (12), a particularly undesirable outcome in children with this blinding eye disease. Whereas two recent studies have found no association between carboplatin treatment and hearing loss in these patients (13, 14), a 5.6% rate of carboplatin ototoxicity has been reported in children treated at other centers (11). This discrepancy may be attributable to variations in schedule and dosing. Etoposide and teniposide are topoisomerase II inhibitors, which have been associated with the induction of secondary leukemias (15). A recent survey
suggests that RB patients treated with these agents may be at increased risk for this devastating complication (16). In 2007, the National Cancer Institute Children’s Oncology Group opened a phase III clinical trial using periocular carboplatin in addition to three-agent chemotherapy and focal therapy for patients with advanced disease. However, local carboplatin therapy has been associated with reduced ocular motility (17), severe pseudo-preseptal cellulitis (18), and blinding optic atrophy (19, 20). The standard alternative for eye salvage in patients with refractory disease is external beam radiation, yet this therapy increases the already high risk of second primary tumors in patients with heritable disease and produces severe midfacial deformities in these children (21). More rational and less toxic therapies for RB patients are clearly required.

HDAC inhibitors (HDACi) are a promising new class of anticancer therapies (22, 23). Many of these agents, including suberoylanilide hydroxamic acid (SAHA), MS-275, PXD101, and LBH589, have been shown to induce cell cycle arrest, activate differentiation programs, and/or mediate apoptosis in...
human cancer cells. One HDACi (SAHA, also known as vorinostat) was recently approved for clinical use, and others are currently being investigated in clinical trials for leukemia, lymphoma, and breast, prostate, ovarian, and other cancers (24, 25). HDACi selectively kill tumor cells (26, 27). The molecular basis for the differential effects of HDACi in tumor versus normal cells is not well understood. Recent work has shown that cancer cells with increased E2F1 activity are highly sensitive to HDACi, which target these cells for apoptosis by promoting E2F1-mediated induction of the proapoptotic gene Bim (28). The inactivation of the RB tumor suppressor gene (RB1) leads to increased E2F activity, and this molecular characteristic of RB cells suggests that HDACi could be a particularly rational therapy for this disease. Karasawa and Okisaka (29) conducted the first investigation of HDAC inhibition in RB cells, in which butyrate and trichostatin A (TSA) increased acetylated histone H3 levels, induced apoptosis, and decreased viability in Y79 cells. In this study, we further evaluated the effects of HDAC inhibition in established and primary human RB cell cultures using three HDACi: TSA, SAHA, and MS-275. We also evaluated the effects of MS-275 in vivo in two rodent models of RB to investigate its potential clinical utility.

Materials and Methods

Cell culture. Y79, Weri-Rb1, and Y79-L1IC human RB cell lines and Rb143 primary human RB cells were cultured in RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin. U2OS and SAOS2 human osteosarcoma cell lines and the Rb3 transgenic murine RB cell line were cultured in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin.

Chemicals and medications. TSA, N-acetylcysteine, and vincristine were purchased from Sigma-Aldrich. SAHA was purchased from Alexis Biochemicals. MS-275 was purchased from Calbiochem. Carboplatin and etoposide were obtained from the inpatient pharmacy at the University of California at San Francisco (San Francisco, CA).

Cell survival assays. For colorimetric analysis, cells were exposed to WST-8 Cell Proliferation Reagent (Djindo), and absorbance was quantified after 2 h using a SpectraMax microplate reader (Molecular Devices). For bioluminescence analysis (Y79-LUC cells only), cells were exposed to 150 μg/mL D-luciferin, and plates were imaged in an IVIS Imaging System (Xenogen) using a 15-s exposure. Luminescence was quantified using Living Image software (Xenogen). For both assays, cell survival was expressed as a percentage of vehicle-treated control values.

Cell cycle analysis. Cells were washed with PBS and fixed in 70% ethanol overnight at 4°C. Cells were then resuspended in 0.38 mmol/L sodium citrate buffer with propidium iodide and RNase and incubated in the dark at 37°C for 30 min. Cell cycle analysis was done using a FACSCalibur flow cytometer (Becton Dickinson) and FlowJo software (Tree Star).

Apoptosis assays. For caspase-3/7 activity assay, cells were exposed to Caspase-Glo 3/7 Reagent (Promega) for 30 min at room temperature, and luminescence was quantified using an LMax luminometer (Molecular Devices). Annexin V assays were done using the Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson). Cells were washed twice with cold PBS and resuspended in binding buffer before addition of Annexin V-FITC and propidium iodide. Cells were...
vortexed and incubated for 15 min in the dark at room temperature before analysis using a FACS Calibur flow cytometer and FlowJo software.

Western blot analysis. Cells or isolated retinal tissues were washed twice with PBS and resuspended in radioimmunoprecipitation assay lysis buffer with protease inhibitor (Roche) before homogenization. Protein concentrations were quantified using the Bradford method (Bio-Rad), and gel electrophoresis was done using 20 μg protein per lane. Protein was transferred onto nitrocellulose membrane and probed with acetyl-histone H3 (Lys9) antibody (Cell Signaling Technology), Histone H3 (Cell Signaling Technology) or α-tubulin (Santa Cruz Biotechnology) antibodies were used as loading controls.

Total RNA isolation and gene expression analysis. Cells were collected and total RNA was isolated using the RNeasy Mini kit (Qiagen). Total RNA concentrations were quantified by spectrophotometry using a SmartSpec Plus (Bio-Rad). cDNA was generated using 1 μg total RNA and the iScript kit (Bio-Rad). Bim expression levels were analyzed by real-time quantitative PCR using the iQ SYBR Green Supermix (Bio-Rad) and a MyiQ Real-time PCR Detection System (Bio-Rad). Fold change in expression was determined by comparative cycle threshold (Ct) analysis with normalization against the housekeeping genes HPRT and GAPDH. The following primers were used: human Bim, GAGATATGGATGGCCACAGA (forward) and CAGCGCGGACATGTACACCTA (reverse); human HPRT, TGACATGGGCTGAACAAATG (forward) and GGTCTCCCTTTCCAGCAAGCT (reverse); and human GAPDH, CATGGTCAATGATGCTACCC (forward) and CATGGGTCCATTGATGAC (reverse).

Reactive oxygen species analysis. Cells were incubated with 20 μmol/L CM-H2DCFDA (Invitrogen) for 30 min at 37°C. Fluorescence was measured using a FACS Calibur flow cytometer, and analysis was done with FlowJo software.

Animals. Two rodent models of RB were used in this study, including the LHβ-Tag transgenic murine model (30) and a recently developed rat ocular xenograft model (31). LHβ-Tag mice carry a transgene composed of the coding region of the SV40 large T antigen (Tag) driven by the promoter of the luteinizing hormone β-subunit gene (LHβ). Tag expression in the retina results in multifocal, bilateral retinal tumors analogous to human RB. For the ocular xenograft model, timed pregnant Wistar rats were purchased from Simonsen Laboratories. Newborn immunonucentive pups were injected in the vitreous of the left eye only with 1,000 luminescence-expressing Y79-LUC cells. Animals were screened for tumor burden at P14 by bioluminescence detection using a Xenogen Imaging Station. All three agents showed potent growth-inhibitory effects in Y79 and Weri-Rb1 cells using the WST-8 colorimetric assay. All three agents showed potent growth-inhibitory effects in Y79 and Weri-Rb1 cells (Fig. 2A). At 72 h after treatment, GI50 values for each agent in Y79 and Weri-Rb1 cells, respectively, were as follows: TSA, 92 nmol/L and 0.72 μmol/L; SAHA, 2.08 and 1.55 μmol/L; and MS-275, 1.34 and 0.98 μmol/L. These values are similar to concentrations required to increase acetyl-histone H3 levels in our initial experiments. We also tested MS-275 in human Rb143 and in transgenic murine Rb3 cells (Fig. 2B). At 72 h, sensitivity of Rb143 cells to MS-275 was similar to that of Y79 and Weri-Rb1 cells (GI50, 0.85 μmol/L), whereas murine Rb3 cells were significantly less sensitive to this agent (GI50, 7.2 μmol/L). We also examined HDACi-mediated growth inhibition using a bioluminescent cell survival assay in Y79-LUC cells, which constitutively expresses the luciferase gene. Y79-LUC cells were exposed to TSA and MS-275 for 72 h and imaged after addition of 150 μg/ml D-luciferin. TSA and MS-275 both showed potent growth-inhibitory effects, with GI50 values similar to those observed in Y79 cells using the WST-8 assay (82 nmol/L for TSA and 1.34 μmol/L for MS-275; Fig. 2C).

Combining HDACi with carboplatin, etoposide, or vincristine results in additive growth-inhibitory effects in RB cells. Combination chemotherapy is usually more effective than mono-drug therapy in the treatment of human cancers. We did cell survival assays to determine the potential clinical utility of MS-275 in combination with standard anti-RB agents. Y79 and Weri-Rb1 cells were treated with a single concentration of MS-275 (0.7 μmol/L for Y79 and 3 μmol/L for Weri-Rb1) plus carboplatin, etoposide, or vincristine over a range of concentrations. In both cell lines, combination treatment resulted in a significant, additive reduction in cell survival relative to cells treated with carboplatin, etoposide, or vincristine alone (Fig. 3A). We also tested carboplatin in combination with TSA or MS-275 using the Y79-LUC bioluminescent cell survival assay. Y79-LUC cells were treated with 100 nmol/L TSA or 1 μmol/L MS-275 with or without carboplatin over a range of concentrations. As in our previous experiments, combination treatment resulted in a significant, additive reduction in cell survival relative to cells treated with carboplatin alone (Fig. 3B).

HDACi induce apoptosis and cell cycle arrest in human RB cells. To further evaluate the cytotoxic effects of HDACi in RB cells, we did a bioluminescent assay for caspase-3/7 activity (late apoptosis marker) in Y79 cells treated with TSA or...
MS-275. Significant increases in caspase activity were observed in cells treated with either agent, relative to controls (Fig. 4A). At 24 h, cells treated with 100 and 200 nmol/L of TSA showed 4.5- and 7.5-fold increases, respectively. Significantly higher increases were observed at 48 h after treatment. In comparison, Y79 cells treated with 5 or 10 μmol/L of MS-275 showed <2-fold increase in caspase activity after 24 h. However, significantly increased activity was observed at 48 h after treatment. Cells treated with 5 and 10 μmol/L of MS-275 showed 5.7- and 5.3-fold increases at this time point, respectively.

Fig. 3. RB cell survival after treatment with HDACi plus carboplatin, etoposide, or vincristine. A, Y79 (left column) or Weri-Rb1 (right column) cells were treated for 48 h with indicated concentrations of carboplatin (CARBO), etoposide (ETO), or vincristine (VIN) with or without MS-275 (0.7 μmol/L for Y79 and 3.0 μmol/L for Weri-Rb1). Cell survival was determined by WST-8 assay. Values are from a representative experiment (n = 4). Bars, SD. A repeat experiment yielded similar results. B, cell survival in carboplatin- and HDACi-treated Y79-LUC cells. Cells were treated for 72 h with carboplatin at the indicated concentrations with or without TSA (100 nmol/L) or MS-275 (1 μmol/L), and luminescence was quantified (n = 12 for TSA, n = 8 for MS-275). Bars, SD. Repeat experiments yielded similar results.
We next examined the effects of MS-275 only on Annexin V translocation (early apoptosis marker) in Y79 cells. Cells were treated with 10 μmol/L MS-275 for 24 h, stained with Annexin V-FITC and propidium iodide, and analyzed by flow cytometry. MS-275–treated cells displayed significant increases in both early and end-stage apoptotic cell populations compared with controls (Fig. 4B). These results suggest that nearly all cells treated with 10 μmol/L MS-275 will undergo cell death, which is consistent with cell survival data in MS-275–treated Y79 cells (Fig. 2A).

We also did cell cycle distribution analysis by flow cytometry in Y79 cells treated with 10 μmol/L MS-275. At 24 h after treatment, cells displayed a significant increase in the G1-phase population and decreases in the S- and G2-phase populations, relative to controls (Fig. 4C). Eighty-two percent of MS-275–treated cells were in G1 phase compared with 55% of cells treated with vehicle alone.

**HDACi-induced apoptosis in RB cells is not mediated by Bim induction.** Other studies have shown that HDACi induce apoptosis in RB1-deficient osteosarcoma cells by promoting...
the induction of Bim, a proapoptotic E2F target gene (28). We began our studies of the mechanisms of HDACi cytotoxicity in RB cells by quantifying Bim expression levels in Y79 cells and in SAOS2 and U2OS osteosarcoma cells after treatment with 100 nmol/L TSA or 1 μmol/L MS-275 for 6 h. Consistent with previous findings, SAOS2 and U2OS cells showed a significant increase in Bim expression after treatment with either TSA or MS-275, but this effect was not observed in Y79 cells (Fig. 5A). Western blot analysis also showed no observable increase in Bim protein levels in TSA-treated Y79 cells relative to controls (data not shown).

MS-275–mediated cytotoxicity is dependent on increased reactive oxygen species levels. HDACi treatment in cancer cells has been shown to increase reactive oxygen species (ROS) levels and induce cell death in a manner dependent on caspase activation (32). We used CM-H2DCFDA labeling and flow cytometry to measure ROS levels in Y79 cells 24 h after MS-275 treatment (Fig. 5B). Cells treated with 1 and 5 μmol/L of MS-275 showed 3.2- and 10.2-fold higher ROS levels than controls, respectively. These increases in ROS levels could be blocked by pretreatment of cells for 2 h with 7.5 mmol/L N-acetylcysteine, a ROS scavenger. This action resulted in rescue of MS-275 cytotoxicity (Fig. 5C). Forty-eight hours after treatment with MS-275, cells pretreated with N-acetylcysteine showed complete resistance to concentrations as high as 5 μmol/L (Fig. 5C).

Local administration of MS-275 to ocular tissues does not cause morphologic changes. We investigated the effects of MS-275 on ocular tissue morphology after intraocular administration in mice. Three groups of three adult wild-type animals received intravitreal injections of 1 μL MS-275 (0.1, 1, and 10 μmol/L) or vehicle only. After 2 days, eyes were sectioned, stained with H&E, and examined by light microscopy. No change in gross ocular anatomy or morphology in ocular tissues was observed by histologic analysis in any group.

Retinal delivery of systemically administered MS-275. Previous animal studies have shown that MS-275 crosses the blood-brain barrier (33). To determine whether MS-275 crosses the blood-retinal barrier, three groups of two adult wild-type mice were treated with 2 or 4 mg MS-275 or vehicle only (i.p.). Four hours after treatment, mice were sacrificed, eyes were enucleated, and retina was surgically isolated. Protein extracts were prepared from isolated retina, and acetylated histone H3 (Lys9) levels were determined by Western blot analysis. Retinal extracts from MS-275-treated mice showed a significant increase in acetylated histone levels relative to controls (Fig. 5A).

MS-275 inhibits RB growth in vivo. We investigated the therapeutic effects of MS-275 in LHβ-Tag transgenic mice and in a rat ocular xenograft model of RB. In the first study, two groups of 14 12-week-old LHβ-Tag mice were treated with 20 mg/kg MS-275 or vehicle (i.p.) every other day for 21 days and sacrificed on day 23. Tumor burden was quantified by histopathologic analysis. Throughout the treatment period, mean body weight of MS-275–treated and control animals did not differ significantly (data not shown). At treatment end point, MS-275–treated animals showed a significant 76% reduction in ocular tumor burden relative to controls (P = 0.036; Fig. 6B). In addition, 5 of 14 MS-275–treated animals had zero tumor burden, whereas all control animals had tumor (Fig. 6C). Figure 6D shows representative sections depicting median tumor burden in MS-275 and control groups (top and middle rows) and tumor regression in MS-275–treated eyes (bottom row).

To establish tumors in the rat ocular xenograft model, newborn immunonaive rat pups were injected intravitreally with Y79-LUC cells in the left eye only. Tumor burden was quantified 2 weeks later by bioluminescent photometry. After baseline screening, 10 animals were pair matched by tumor burden and treated with 20 mg/kg MS-275 or vehicle only (i.p.) every other day for 13 days. At completion of treatment on day 13, we observed a significant, 63% reduction in mean
tumor burden in the MS-275–treated group compared with the vehicle-treated control group ($P = 0.039$; Fig. 6E). Furthermore, each individual MS-275–treated animal possessed lower tumor burden than its pair-matched control animal (Fig. 6F).

**Discussion**

In developed countries, the survival rate among children with intraocular RB is ~95%. However, children who survive this
Out of the many novel classes of anticancer therapies currently in development, we chose to evaluate the potential utility HDACi for the treatment of RB. Previous research has identified several characteristics of HDACi that suggest that they would be particularly rational therapies for this disease. These agents induce tumor-selective cytotoxic effects (26, 27) and they particularly target tumor cells with deregulated E2F activity (28, 34). Increased E2F activity is a molecular consequence of loss of RB protein function (35), the signature characteristic of RB cells. Furthermore, HDACi are agents that modify gene expression via epigenetic regulation, and RB cells have marked changes in their epigenetic profile (36–39), which are thought to contribute to tumor progression (40). Finally, the antitumor effects of HDACi are synergistic with radiation and with agents currently used to treat RB (41–44). It is therefore possible that the efficacy of current regimens for this disease could be improved by coadministration of a HDACi.

In the present study, we evaluated the growth-inhibitory effects of three first-generation HDACi in RB cell lines. Among the three HDACi tested (TSA, SAHA, and MS-275), we selected MS-275 for further investigation in vitro (apoptosis and cell cycle analyses; drug combination assays) and in vivo (in a transgenic murine and a rat ocular xenograft rat model of RB). Our selection of MS-275 was based on its high therapeutic index, promising results in clinical trials, and availability in amounts sufficient for animal studies. Our animal studies show that MS-275 is a well-tolerated and highly effective treatment for RB in vivo. MS-275 did not alter ocular tissue morphology after intraocular presentation. It readily penetrated the retina after systemic administration, and it significantly reduced tumor burden in both rodent models.

Phase I and II clinical trials have shown that MS-275 also has limited toxicity in humans (45, 46). No grade 4 toxicities were found in patients in a phase I trial of MS-275 administered weekly to patients with refractory solid tumor and lymphoid malignancies (46). The most severe toxicities observed in this trial were reversible or non–dose-limiting grade 3 hypoalbuminemia, hyponatremia, hypophosphatemia, and myelosuppression. MS-275 was also well tolerated in a phase II trial for metastatic melanoma (47). Weekly oral administration of MS-275 resulted in nonobjective tumor stabilization at metastatic locations along with minor grade 1 to 2 diarrhea, nausea, and hypophosphatemia. These data, along with our findings in RB cells that MS-275 induces significant, additive cytotoxic effects when administered with standard anti-RB agents, suggest that HDAC would be a well-tolerated and effective addition to current chemotherapeutic regimens for this disease.

Additional investigation into the mechanisms for selective cytotoxicity of HDACi in RB and other tumor cells is warranted. We observed increased acetylated histone protein levels in both RB cells and normal retinal cells exposed to HDACi. Thus, the underlying reason for high therapeutic index of these agents remains enigmatic. Previous studies have shown that HDACi increase ROS levels in neoplastic cells but not in normal cells (26). Increased ROS levels in HDACi-treated tumor cells could in turn be explained by the finding that HDACi increase levels of thioredoxin, a cellular antioxidant, in normal but not in transformed cells (32). In this study, we found that HDACi cytotoxicity could be rescued in RB cells by antioxidant pretreatment, which may mimic the intrinsic mechanism protecting normal cells. Ungerstedt et al. (32) also found that inhibition of caspase activity did not block HDACi-induced cell death mediated by increased ROS levels. More analysis of this caspase-independent pathway and thioredoxin regulation in RB cells could provide additional insight into the potential responsiveness of RB tumors to HDACi therapy.

Changes in gene expression and regulation may also contribute to the high sensitivity of tumor cells to HDACi. A recent study in RB1-deficient osteosarcoma cells showed that HDACi promote induction of the proapoptotic E2F1 target gene Bim in these cells (28). The authors of this study suggest that this action could explain the heightened sensitivity of
RB/E2F pathway-deregulated tumor cells to HDACi. However, we observed no up-regulation of *Bim* expression following HDACi treatment of RB cells, which universally express the *Bcl-x* gene. This up-regulation of *Bim* expression following HDACi treatment of RB cells suggests that RB cells are resistant to TRAIL-mediated apoptosis due to silencing of caspase-8 expression. Demethylating agents were able to restore RB cell sensitivity to TRAIL treatment. HDACi may also restore or mediate death receptor pathway activation in RB cells, and these properties should be investigated. Finally, inactivation of other HDACi agents in RB, most notably SAHA, LBH569, and valproic acid, should also be conducted. The selectivity of these agents for HDAC isoforms, their pharmacologic profiles, and their therapeutic indices vary, and further work would provide useful information for selection of an optimal agent for the treatment of RB.

The antitumor effects of MS-275 observed in our *in vitro* and preclinical animal studies are a promising indication that HDACi could provide significant clinical benefits in the treatment of RB. The inclusion of HDACi in combination chemotherapeutic regimens could enable dose reduction of standard broad-spectrum agents, reduction of toxicity, and improved vision retention in children with this disease.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Michael A. Dyer, Ph.D., of St. Jude Children's Research Hospital for his generous gift of Y79-LUC cells.

**References**

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*Clin Cancer Res* 2008;14:3113-3123.

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