Targeting the p53 Pathway in Retinoblastoma with Subconjunctival Nutlin-3a

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Abstract

Retinoblastoma is a rare childhood cancer of the retina that begins in utero and is diagnosed in the first years of life. The goals of retinoblastoma treatment are ocular salvage, vision preservation, and reduction of short- and long-term side effects without risking mortality because of tumor dissemination. To identify better chemotherapeutic combinations for the treatment of retinoblastoma, several groups have developed genetic mouse models and orthotopic xenograft models of human retinoblastoma for preclinical testing. Previous studies have implicated the MDMX protein in the suppression of the p53 pathway in retinoblastoma and shown that the MDM2/MDMX antagonist, Nutlin-3a, can efficiently induce p53-mediated cell death in retinoblastoma cell lines. However, Nutlin-3a cannot be administered systemically to treat retinoblastoma, because it has poor penetration across the blood–ocular barrier. Therefore, we developed an ocular formulation of Nutlin-3a, Nutlin-3aOC, and tested the pharmacokinetics and efficacy of this new formulation in genetic and human retinoblastoma orthotopic xenograft models of retinoblastoma. Here, we show that Nutlin-3aOC specifically and efficiently targets the p53 pathway and that the combination of Nutlin-3aOC with systemic topotecan is a significantly better treatment for retinoblastoma than currently used chemotherapy in human orthotopic xenografts. Our studies provide a new standardized approach to evaluate and prioritize novel agents for incorporation into future clinical trials for retinoblastoma. Cancer Res; 71(12); 4205–13. © 2011 AACR.

Introduction

Each year, approximately 250 to 300 cases of retinoblastoma are diagnosed in the United States and 5,000 cases are diagnosed worldwide (1). The primary goals of therapy are cure and ocular salvage; however, enucleation remains a frequent treatment for advanced intraocular disease. In developing countries, patients more often present with advanced disease and the long-term survival rate is approximately 50% (2). The most widely used chemotherapy treatment regimen for retinoblastoma includes systemic administration of etoposide (ETO), carboplatin (CBP), and vincristine (VCR), and for advanced disease, we need to develop localized therapies that do not lead to overwhelming systemic or ocular toxicity. In this study, we sought to test the efficacy of locally delivered targeted chemotherapy for retinoblastoma in preclinical models. One target that is tractable in retinoblastoma is the p53 pathway. The p53 gene is intact in retinoblastoma, and the pathway is silenced by increased expression of the upstream regulator MDMX (7, 8). Nutlin-3a is one of the first selective small molecule inhibitors of the MDM2–p53 interaction (9); it also partially inhibits the MDMX–p53 interaction at higher concentrations (8) and can efficiently...
kill retinoblastoma cell lines in vitro and in vivo (8, 10). Recent pharmacokinetic (PK) studies of oral and i.v. Nutlin-3a formulations (11, 12) have shown that Nutlin-3a has poor penetration into the vitreous by using these routes of administration.

To improve intraocular penetration of Nutlin-3a, we developed an ocular formulation of Nutlin-3a by using Food and Drug Administration (FDA)-approved adjuvants (Nutlin-3aOC), and subconjunctival administration of Nutlin-3aOC led to a 2,000- to 20,000-fold increase in the intraocular penetration. There were no adverse effects related to vision, intraocular pressure (IOP), or blood cell counts following multiple subconjunctival doses of Nutlin-3aOC in adult mice. Preclinical testing of Nutlin-3aOC combined with TPT3m in our MDMX (Chx10-Cre;Rblm1 Δ17; p107 Δ7;MDMXlox/lox) and p53TKO (Chx10-Cre;Rblm1 Δ17; p107 Δ7; p53lox/lox) genetic mouse models of retinoblastoma provided genetic validation of targeting the p53 pathway in vivo. Preclinical testing of Nutlin-3aOC combined with TPT3m in human retinoblastoma orthotopic xenograft models showed significant improvement in outcome compared with all currently available therapies for the treatment of retinoblastoma. This study establishes a standardized protocol for testing new agents in preclinical models for retinoblastoma and highlights the value of combinatorial target and broad-spectrum chemotherapy in cancer treatment.

Materials and Methods

Genetic mouse models of retinoblastoma and orthotopic murine xenografts

The 2 genetic mouse models of retinoblastoma (Chx10-Cre; Rb1Δ17; p107 Δ7; p53Δlox/lox) and Chx10-Cre;Rb1Δ17; p107 Δ7; p53Δlox/lox) have been described previously (7, 13). The preparation and injection of human primary tumor specimens into the eyes of severe combined immunodeficient mice (SCID) mice (B6.CB17-Prkdcscid/scid > SCID > Szj; The Jackson Laboratory) has also been described elsewhere (7). All animal care and experimental procedures used in this study were approved by the Animal Care and Use Committee at St. Jude Children’s Research Hospital.

Preclinical testing

All preclinical testing procedures have been described previously (6). VCR/ETO/CBP combination therapy was administered systemically over a 3-day course as follows: VCR 0.38 mg/kg per dose, intraperitoneally (i.p.) and CBP (80 mg/kg per dose, i.p.) were administered on day 1, and ETO (6 mg/kg per dose, i.p.) was administered on days 1 to 3. The CBPsubconj/TPT3m combination therapy was administered over a 5-day course as follows: CBPsubconj (100 µg/eye) on day 1, and TPT3m (0.7 mg/kg per dose, i.p.) on days 1 to 5. The Nutlin-3aOC/TPT3m combination therapy was administered over a 5-day course as follows: 25 mmol/L Nutlin-3aOC (10 µL/eye) on day 1, and TPT3m (0.7 mg/kg per dose, i.p.) on days 1 to 5.

Animals that underwent enucleation because of an elevated IOP (IOP > 14) were scored as having progressive disease (PD). The remaining animals (IOP ≤ 13) were graded as complete response (CR) if end-of-therapy optometry showed vision greater than or equal to 0.15 cycles/degree and as stable disease (SD) if end-of-therapy optometry showed vision less than or equal to 0.14 cycles/degree.

Statistical methods

Event-free survival (EFS) of xenograft models was defined as the period from the start of chemotherapy to the day a mouse was euthanized because of elevated IOP in at least 1 eye. An individual mouse served as the unit of analysis. No competing events were observed. The Kaplan–Meier method was used to estimate EFS distributions. Exact log-rank tests were used to compare survival distributions among treatment groups. The eye was the unit of analysis for the genetic model. In these studies, we wanted to account for the correlation of outcome within an animal, so the primary analysis was time-to-first-event in an animal, where an event was defined as the enucleation of the eye because of the elevated IOP. Some eyes were not followed for the duration of the study for reasons other than enucleation because of the elevated IOP (e.g., death during anesthesia, moribund because of secondary tumors, and so on); these were considered competing events. The cumulative incidence of enucleation because of the elevated IOP was estimated for each treatment group and mouse strain. Gray’s test was used to compare cumulative incidences among groups.

Ocular histopathology

After enucleation, eyes were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated through an alcohol series, and washed in xylene. They were then embedded in paraffin, and 5-µm sagittal sections were cut through the optic nerve for histopathologic analysis.

Nutlin-3a PKs

Adult C57Bl/6 mice were treated with 25 mmol/L Nutlin-3aOC (10 µL) in each eye. At serial time points (preinjection and 0.25, 0.5, 1.5, 4, and 6 hours postinjection in triplicate), a cardiac puncture was conducted; blood was collected; and plasma was isolated. Once the cardiac puncture was completed, the animal was euthanized by cervical dislocation; the eyes were removed, and the vitreous and retinae were collected and flash frozen for later analysis. The methods for Nutlin-3a detection have been described elsewhere (11).

Results

Preclinical testing in 2 genetic models of retinoblastoma

The development and characterization of preclinical genetic models of retinoblastoma (6, 14) provide a unique opportunity to begin to test the efficacy of new combinations of chemotherapy and compare those novel treatments with current therapeutic regimens. Two complementary genetic models (MDMX and p53TKO) of retinoblastoma with p53-pathway inactivation develop aggressive bilateral disease. The initiation, progression, invasion, and histopathologic features of retinoblastoma in p53TKO and MDMX strains are indistinguishable (Fig. 1A–C; ref. 7). We have previously
Figure 1. Efficacy of TPT/CBP and VCR/ETO/CBP in genetic models of retinoblastoma. A, photograph of a p53TKO mouse retinoblastoma (arrow). B, hematoxylin and eosin (H&E) staining of advanced-stage p53TKO retinoblastoma. C, step plot of age to moribund status for Chx10-Cre;RbLox/Lox;p107Lox/Lox (p53TKO), Chx10-Cre;RbLox/Lox;p107Lox/Lox;MDMXTg (MDMX), and Chx10-Cre;RbLox/Lox;p107Lox/Lox;p53Lox/Lox (p53TKO) mice. D, retinal camera/fluorescein angiography images of control (C57Bl/6) and MDMX retinoblastoma at diagnosis (arrow). E, chemotherapy schedule for 1 course of therapy. F and G, histogram of responses of MDMX and p53TKO models to both chemotherapeutic regimens as CR, SD, and PD. H and I, step plots of time to moribund status for MDMX and p53TKO mice. J, histogram of response to therapy in eyes based on IOP. K–N, H&E staining of eyes that showed PD (K and L) and for 1 that showed SD (M and N). Arrows indicate viable tumor cells and open arrowhead indicates necrotic cells. on, optic nerve. Scale bars: B, 0.5 mm; K and M, 25 μm; L and N, 10 μm.
described a preclinical protocol for testing chemotherapy in the p53TKO strain (6). Briefly, retinoblastoma is diagnosed with a digital retinal camera and fluorescein angiography at 5 to 10 weeks of age (Fig. 1D). Following a baseline analysis of blood counts/chemistries, visual acuity, and IOP, the chemotherapy protocol is initiated. The dosages of each anticancer drug used in the mice were chosen to yield systemic exposures similar to those observed in children treated with the same anticancer drug. The schedule of administration is identical to that used in pediatric clinical protocols (Fig. 1E).

To test the efficacy of the VCR/ETO/CBP combination and the CBPsubconj/TPTsyst combination in our genetic mouse models of retinoblastoma, we treated 93 p53TKO mice (final analysis; 183 eyes) and 54 MDMX mice (final analysis; 106 eyes). Assessments of all mice were initiated before 12 weeks of age. Overall, 66% (61/93) of the p53TKO mice completed 6 courses of chemotherapy (18 weeks on study), and 98% (53/54) of the MDMX mice completed 6 courses of chemotherapy. The MDMX mice responded better to the VCR/ETO/CBP therapy than did the p53TKO mice (P < 0.0001), but there was no significant difference in the responses of the 2 strains to the CBPsubconj/TPTsyst treatment (Fig. 1F–I). There was also no difference in response to CBPsubconj/TPTsyst versus VCR/ETO/CBP for either strain (P = 0.0812 for MDMX, and P = 0.6894 for p53TKO). We noticed a striking correlation between IOP and outcome in this study. Specifically, for either strain on either treatment regimen, the tumors typically progressed once the IOP reached 15 mmHg or higher (Fig. 1J). Histopathologic analysis of each eye confirmed the response to therapy (Fig. 1K–I). Representative MBI ultrasound, and visual acuity testing data for each response category are shown Supplementary Fig. S1.

Improved solubility of Nutlin-3a in an ocular formulation

Previous studies have shown that Nutlin-3a can block the MDM2–p53 interaction and, to a lesser extent, the MDMX–p53 interaction (8, 15), but there is a poor intraocular penetration of nutlin-3a following systemic administration (11, 12). We developed an ocular formulation of Nutlin-3a (Nutlin-3aOC) by using FDA-approved adjuvants for ocular delivery in the appropriate ratios. We identified 5 organic solvents out of 86 FDA-approved ingredients for further optimization (Supplementary Table S1). Cremophor EL (polyoxyl 35 castor oil) was essential to maintain maximum concentrations of Nutlin-3a in suspension (Supplementary Table S1). We carried out a PK study in adult C57Bl/6 mice. We administered 10 µL of 25 mmol/L Nutlin-3aOC subconjunctivally to both eyes and then harvested plasma and vitreous from each animal at 5 different time points (0.5, 1, 2, 4, and 8 hours). The plasma and vitreous systemic exposure [(measured by area under the concentration–time curve (AUC)] after subconjunctival administration was compared with that observed after 200 mg/kg oral (Fig. 2A) and 10 mg/kg i.v. delivery (Fig. 2B). The AUC ratio of vitreous to plasma for subconjunctival administration (28.6) was approximately 2,000-fold greater than that observed for oral delivery. The AUC ratio of vitreous to plasma for subconjunctival administration (28.6) was approximately 2,000-fold greater than that observed for oral delivery.

Subconjunctival delivery of Nutlin-3aOC improves intraocular penetration

To study the intraocular penetration of Nutlin-3aOC, we carried out a PK study in adult C57Bl/6 mice. We administered 10 µL of 25 mmol/L Nutlin-3aOC subconjunctivally to both eyes and then harvested plasma and vitreous from each animal at 5 different time points (0.5, 1, 2, 4, and 8 hours). The plasma and vitreous systemic exposure [(measured by area under the concentration–time curve (AUC)] after subconjunctival administration was compared with that observed after 200 mg/kg oral (Fig. 2A) and 10 mg/kg i.v. delivery (Fig. 2B). The AUC ratio of vitreous to plasma for subconjunctival administration (28.6) was approximately 2,000-fold greater than that observed for oral delivery. The AUC ratio of vitreous to plasma for subconjunctival administration (28.6) was approximately 2,000-fold greater than that observed for oral delivery.

![Figure 2. Subconjunctival delivery of Nutlin-3a improves intraocular penetration. A–C, the concentration of Nutlin-3a was measured at 0.5, 1.0, 2.0, 4.0, and 8.0 hours in plasma (black line) and vitreous (green line). The concentration versus time plot was used to fit a 2-compartment model to determine the AUC and calculate the ratio of the AUC in vitreous/plasma for each route of delivery.](image-url)
higher than that observed after oral administration and 20,000-fold higher than i.v. administration (Fig. 2C).

**Lack of ocular and hematologic toxicity associated with Nutlin-3aOC**

We tested Nutlin-3aOC for acute ocular toxicity in a cohort of 10 adult C57Bl/6 mice. A single subconjunctival injection of Nutlin-3aOC was administered in 1 eye. Visual acuity and IOP were then monitored repeatedly over the next 10 days (Supplementary Fig. S2A and B). A matched cohort of mice received subconjunctival injections of vehicle (15% pPPG, 10% PPG, 5% cremophor el., and 70% PBS) alone. We found no change in vision or IOP for either Nutlin-3aOC or vehicle and no change in blood counts (complete blood count with differential (CBC-D); data not shown). To analyze any possible hematopoietic toxicity of Nutlin-3aOC in greater detail, we administered 6 weekly subconjunctival injections of Nutlin-3aOC (10 µL) in 15 C57Bl/6 adult mice. Three additional mice received vehicle alone. A CBC-D was carried out daily for 5 days following each dose of Nutlin-3aOC. We found no significant differences in the white blood cell count (WBC), hemoglobin (Hb), or platelets (Plts) during the 6 weeks of testing (Supplementary Fig. S2C–E).

Recent preclinical retinoblastoma studies have shown that TPT is an effective treatment (6), and this agent is currently being tested in patients with retinoblastoma (RET5 protocol). Because TPT has a good intraocular penetration following systemic administration in murine models of retinoblastoma (6), we tested the toxicity of Nutlin-3aOC combined with TPTsys. We treated 4 C57Bl/6 adult mice with Nutlin-3aOC on day 1 and TPTsys on days 1 to 5 every 3 weeks for 18 weeks (6 courses). We found no significant differences in the white blood cell count (WBC), hemoglobin (Hb), or platelets (Plts) during the 6 weeks of testing (Supplementary Fig. S2C–E).

To compare the efficacy of alternating courses of CBPsubconj/TPTsys with Nutlin-3aOC/TPTsys in the orthotopic xenografts with that seen in the genetic mouse models, we injected SJ-39 retinoblastoma cells into the vitreous of both eyes of 100 immunocompromised mice: 30 were untreated, 34 received VCR/ETO/CBP, 21 received CBPsubconj/TPTsys, and 15 received CBPsubconj/TPTsys alternating with Nutlin-3aOC/TPTsys. The dosages, schedules, routes of administration, and monitoring were identical to those used in our genetic mouse models of retinoblastoma. Although the tumor initiation, invasion, and histopathologic features of the xenograft retinoblastoma are very similar to those of the genetic models previously described, the tumors are more aggressive, with shorter time to morbidity from disease than either genetic model (compare Fig. S5 with Fig. 1C). We found a slight improvement in outcome from VCR/ETO/CBP (P = 0.0002;
Fig. 5E), but not from CBPsubconj/TPTsyst (P = 0.1581; Fig. 5F).

The CBPsubconj/TPTsyst with Nutlin-3aOC/TPTsyst treatment showed a significantly better response (P < 0.0001; Fig. 5G–I). MRI, histopathologic analysis, and molecular diagnostics confirmed that the p53 pathway was activated in these tumors leading to apoptosis (Fig. 5N). The immunoblot experiments were carried out on tumors from animals that received Nutlin-3aOC as a single agent without TPTsyst to confirm molecular targeting in vivo.

Discussion

Here, we present the most comprehensive preclinical study to date for retinoblastoma incorporating broad-spectrum chemotherapy and molecular-targeted therapy in genetic and orthotopic xenograft models of this devastating childhood cancer. We achieved good response in the p53TKO and MDMX mouse models for 2 clinically relevant chemotherapy combinations, VCR/ETO/CBP and CBPsubconj/TPTsyst. To explore targeted therapy for retinoblastoma, we developed an ocular formulation of Nutlin-3a (Nutlin-3aOC), and subconjunctival delivery in our preclinical models of Nutlin-3aOC improved intraocular penetration 2,000- to 20,000-fold. The combination of Nutlin-3aOC with TPTsyst was an effective treatment in the MDMX retinoblastoma mice, but it was much less effective in the p53TKO mice, thereby providing direct genetic confirmation of targeting the p53 pathway in vivo. In sharp contrast to the currently available chemotherapy regimens, Nutlin-3aOC/TPTsyst showed a significant improvement in outcome in our human retinoblastoma orthotopic xenograft model. These
data can be used to direct future efforts to target the p53 pathway in retinoblastoma clinical trials.

**Genetic mouse models of retinoblastoma**

The p53TKO and MDMX mouse models of retinoblastoma have virtually indistinguishable penetrance and disease progression. Therefore, they provide an ideal pair of strains to test the efficacy of targeting the p53 pathway in vivo. Agents that target the MDMX–p53 interaction are expected to show efficacy in the MDMX strain but be much less effective in the p53TKO strain. This is exactly what we found with Nutlin-3aOC. The MDMX mice showed an overall good response to therapy with Nutlin-3aOC/TPTsyst whereas the p53TKO mice responded very poorly, with 78% of eyes succumbing to PD. These striking results confirm the direct targeting of the p53 pathway in vivo by using Nutlin-3a. We also validated targeting of the MDMX–p53 interaction biochemically by showing that downstream targets of the p53 pathway were upregulated. Therefore, genetic mouse models can serve as useful tools to validate molecular targeted therapies if they are well characterized and incorporated in preclinical studies that recapitulate clinical trials in patients.

**Comprehensive preclinical testing**

It is important to incorporate clinically relevant imaging and diagnostic modalities into preclinical studies because they can provide a better understanding of disease progression and side effects of treatment and lead to new insights into the biology of tumorigenesis. One example presented here was the striking correlation between elevated IOP and disease progression. Over 95% of eyes with IOP more than 15 mmHg failed therapy. It is reasonable to postulate that IOP is a reliable proxy for disease burden in retinoblastoma as advanced-stage eyes are more likely to have elevated IOP than early-stage eyes. However, there is no perfect correlation between tumor burden and IOP (Brennan and Dyer, unpublished data) as there are some examples of eyes completely filled with tumor with normal IOP and others with lower tumor burden and elevated IOP. This may suggest that the circulation of the vitreous can be disrupted as a result of retinoblastoma progression, and the particular site of tumor growth and dissemination may be reflected by the elevated IOP and as a result, poor response. In our study, we cannot distinguish between these 2 possibilities (disease burden versus vitreal circulation) but they provide new hypotheses to test in future studies and will likely shed light on important aspects of retinoblastoma biology.

Of equal importance is the dose and schedule for drug delivery in preclinical models. If the dose and/or schedule are different from those used clinically, then it is virtually impossible to directly compare the efficacy of new agents to existing regimens. We have found that weight-converted dosing from...
children to mice can be very misleading when exposure (AUC) is measured. Therefore, all of our agents were dosed in rodents to match the AUC in patients. We also precisely matched the schedule of drug administration as this can have a profound effect on efficacy and toxicity. The schedule of delivery is particularly important for subconjunctival administration in patients with retinoblastoma because children with retinoblastoma can receive a subconjunctival injection only once every 3 weeks when they undergo examination under anesthesia. The only time we used a more accelerated schedule of Nutlin-3aOC was when we were studying any potential hematopoietic side effects. Following a single Nutlin-3aOC injection, we failed to detect any change in results from a CBC-D, so we carried out a follow-up study with once-weekly Nutlin-3aOC for 6 weeks. Even with this accelerated schedule, we could not detect any ocular or hematopoietic toxicity of Nutlin-3aOC.

**Targeting the p53 pathway in cancer**

The tumor suppressor protein p53 provides protection from cellular insults and malignant transformation (16). In retinoblastoma, an intact p53 gene is silenced by overexpression of MDMX (8). Inhibition of the p53–MDMX interaction in retinoblastoma cells in culture and in vivo by Nutlin-3a (9) provides a unique opportunity to develop locally delivered and targeted chemotherapy for retinoblastoma. Although Nutlin-3a selectively inhibits p53–MDM2 as compared with p53–MDMX, it does disrupt the p53–MDMX interaction in retinoblastoma cells and is particularly effective when combined with TPT (8). There are no detectable levels of MDM2 in retinoblastoma cells, but it is possible that low levels of MDM2 are present and inhibition of both MDM2 and MDMX is important for the cytotoxic effect in retinoblastoma.

Nutlin-3a was first described 7 years ago (9), yet the cost and limited availability of Nutlin-3a hindered any further preclinical testing of this compound. To overcome this significant obstacle to translational research for pediatric cancer, we synthesized 10 g of Nutlin-3a for use in PK (refs. 11, 12) and preclinical efficacy studies. Intracocular penetration of Nutlin-3a following oral or i.v. administration was very poor (11, 12), so we developed an ocular formulation of Nutlin-3a OC.

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**Figure 5.** Improved outcomes for mice with human orthotopic retinoblastoma xenografts following Nutlin-3aOC/TPT treatment. A, photograph of the SJ-39 orthotopic xenograft with advanced retinoblastoma (arrow). B, retinal camera image and fluorescein angiography (C) of a retinoblastoma xenograft at initiation of therapy. D, H&E staining of a paraffin section of a xenograft at the moribund endpoint in an untreated animal. E, step plot for orthotopic xenografts receiving no treatment, as compared with those receiving VCR/ETO/CBP or CBPsubconj/TPTsubconj (F) or Nutlin-3aOC/TPT (G). H and I, T1-weighted MRI images of an eye treated with Nutlin-3aOC/TPT (H) or untreated control eye. J–M, representative H&E staining of a paraffin section from eyes that showed PD in untreated animals (J and K) and for 1 that showed extensive necrosis after treatment with Nutlin-3aOC (L and M). Arrows indicate viable cells, and the open arrowhead indicates a dying cell. N, immunoblot of p21 and β-actin expression in retinoblastoma xenograft 24 hours after Nutlin-3aOC or vehicle treatment in vivo. TPT was not administered for the immunoblot studies. AC, anterior chamber; C, cornea; L, lens; ON, optic nerve; Sc, sclera. Scale bars: J and L, 25 μm; K and M, 10 μm.
and tested the PKs of Nutlin-3aOC following subconjunctival administration. There was a dramatic increase (2,000- to 20,000-fold increase in vitreal/plasma AUC) in intraocular penetration of Nutlin-3a following subconjunctival delivery of Nutlin-3aOC. Toxicity studies showed that subconjunctival delivery of Nutlin-3aOC was well tolerated without ocular or hematologic systemic toxicity. However, single-agent therapy is not clinically relevant in the treatment of pediatric retinoblastoma. Therefore, Nutlin-3aOC was combined with TPT\textsuperscript{ sys} in an alternating schedule with CBP\textsubscript{subconj}/TPT\textsuperscript{ sys}.

A recent study has shown that Nutlin-3 can inhibit Pgp, and this may in turn increase systemic exposure of anticancer chemotherapeutic agents that are substrates for Pgp (17). It is unlikely that this is the primary mechanism for the antitumor effects observed in our MDMX genetic model of retinoblastoma or our orthotopic xenografts because PKs showed that the sustained systemic exposure of Nutlin-3a was very low during the 5 days when TPT was administered.

**Orthotopic xenografts**

Primary human retinoblastomas do not grow well in the flank of immunocompromised mice, and the drug penetration in the flank will be very different from the eye. Therefore, we developed orthotopic xenografting methods for retinoblastoma with 100% engraftment in the vitreous of the eye of immunocompromised mice (Flores-Otero and Dyer, unpublished data). The cellular, molecular, and genetic properties of the human primary tumor (SJ-39), including high levels of MDMX mRNA and protein, are preserved in the subsequent passages of the orthotopic xenograft (7). The growth of this tumor sample is more aggressive than the genetic model with rapid growth and invasion in the anterior chamber, choroids, and optic nerve. This may explain why VCR/ETO/CRP or CBP\textsubscript{subconj}/TPT\textsuperscript{ sys} regimens had little effect in the xenograft model but were essentially curative in the genetic mouse models. These results emphasize the importance of using both genetic models and orthotopic xenografts in preclinical testing. By integrating preclinical studies across experimental paradigms, we hope to gain great insight into the efficacy of new therapeutic agents.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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