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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 due to 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-3a^{OC}, 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-3a^{OC} specifically and efficiently targets the p53 pathway and that the combination of nutlin-3a^{OC} 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.

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 ~50% (2). The most widely used chemotherapy treatment regimen for retinoblastoma includes systemic administration of etoposide (ETO), carboplatin (CBP) and vincristin (VCR) and for early stage disease it is possible to eliminate ETO without affecting outcomes (3). Ongoing clinical trials (RET5 protocol) are focused on incorporating systemic topotecan (TPT) into the currently used treatment regimens.

The eye provides a unique opportunity for local, concentrated delivery of specific chemotherapeutic drugs in order to minimize systemic exposure and side effects. Previously, both CBP (4) and topotecan (TPT) (5) have been tested in retinoblastoma patients using subconjunctival delivery. A recent preclinical study compared the efficacy and toxicity of the combination of subconjunctival TPT (TPT^{subconj}) with systemic CBP to subconjunctival CBP (CBP^{subconj}) with systemic TPT (6). The latter combination had the best efficacy and least toxicity in preclinical models. However, the local, periocular inflammation associated with CBP^{subconj} may limit its widespread use in children with retinoblastoma.

To improve ocular salvage for retinoblastoma patients with advanced disease, we need to develop localized therapies that do not lead to overwhelming systemic or ocular toxicity. However, testing new agents is very challenging in clinical trials, because the overall number of patients is small. Therefore, preclinical testing is of particular importance to identify drug combinations that have the best chance of improving the outcome of patients with retinoblastoma without debilitating side effects. 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 studies of oral and intravenous nutlin-3a formulations (11, 12) have shown that nutlin-3a has poor penetration into the vitreous using these routes of administration.

To improve intraocular penetration of nutlin-3a, we developed an ocular formulation of nutlin-3a using FDA approved adjuvants (nutlin-3a^{OC}) and subconjunctival administration of nutlin-3a^{OC} led to a 2,000–20,000 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-3a^{OC} in adult mice. Preclinical testing of nutlin-3a^{OC} combined with systemic topotecan in our MDMX (*Chx10-Cre;Rb^{Lox/Lox};p107^{-/-};MDMX^{Tg}*) and p53TKO (*Chx10-Cre;Rb^{Lox/Lox};p107^{-/-};p53^{Lox/Lox}*) genetic mouse models of retinoblastoma provided genetic validation of targeting the p53 pathway in vivo. Preclinical testing of nutlin-3a^{OC} combined with systemic topotecan in human retinoblastoma orthotopic xenograft models showed significant improvement in outcome compared to 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 combining 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;Rb^{Lox/Lox};p107^{-/-};MDMX^{Tg}* and *Chx10-(6)Cre;Rb^{Lox/Lox};p107^{-/-};p53^{Lox/Lox}*) have been described previously (7, 13).

The preparation and injection of human primary tumor specimens into the eyes of SCID mice (B6. CB17-Prkdc^{scid}SzJ; Jackson Labs, Bar Harbor, ME) 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, IP) and CBP (80 mg/kg per dose, IP) were administered on Day 1, and ETO (6 mg/kg per dose, IP) was administered on Days 1–3. The CBP^{subconj}/TPT^{syst} combination therapy was administered over a 5-day course as follows: CBP^{subconj} (100 µg/eye) on Day 1, and TPT^{syst} (0.7 mg/kg per dose, IP) on Days 1–5. The nutlin-3a^{OC}/TPT^{syst} combination therapy was administered over a 5-day course as follows: 25 mM nutlin-3a^{OC} (10 µL/eye) on Day 1, and TPT^{syst} (0.7 mg/kg per dose, IP) on Days 1–5.

Animals who underwent enucleation due to elevated IOP (IOP >14) were scored as progressive disease (PD). The remaining animals (IOP ≤13) were scored as complete response (CR) if end-of-therapy optometry demonstrated vision greater than or equal to 0.15 cycles/degree and as stable disease (SD) if end-of-therapy optometry demonstrated 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 due to elevated IOP in at least 1 eye. An individual mouse served as the unit of analysis. There were no competing events observed. The method of Kaplan and Meier 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 due to elevated IOP. Some eyes were not followed for the duration of the study for reasons other than enucleation due to elevated IOP (e.g., death during anesthesia, moribund due to secondary tumors etc.); these were considered competing events. The cumulative incidence of enucleation due to 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 Pharmacokinetics

Adult C57Bl/6 mice were treated with 25-mM nutlin-3a^{OC} (10 µL) in each eye. At serial time points (preinjection and 0.25, 0.5, 1.5, 4, and 6 h postinjection in triplicate), a cardiac puncture was performed; 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 Two Genetic Models of Retinoblastoma

The development and characterization of preclinical genetic models of retinoblastoma (6, 14) provides 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 the p53TKO and MDMX strains are indistinguishable (Fig. 1A–C and (7)). We have previously 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 that 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 vincristin/etoposide/carboplatin (VCR/ETO/CBP) combination and the subconjunctival carboplatin/systemic topotecan (CBP_{subconj}/TPT^{syst}) 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 CBP_{subconj}/TPT^{syst} treatment (Fig. 1F–I). There was also no difference in response to CBP_{subconj}/TPT^{syst} 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 mm Hg or higher (Fig. 1J). Histopathologic analysis of each eye confirmed the response to therapy (Fig. 1K–N). Representative MRI, ultrasound, and visual acuity testing data for each response category are shown Supplemental Fig. 1.

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 poor intraocular penetration of nutlin-3a following systemic administration (11, 12). We developed an ocular formulation of nutlin-3a (nutlin-3a^{OC}) 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 (Sup. Table 1). Cremophor eL (polyoxyl 35 castor oil) was essential to maintain maximum concentrations of nutlin-3a in suspension (Sup. Table 1). By further manipulating other key ingredients (Sup. Table 1) and utilizing a probe sonicator, we achieved the maximum concentration of nutlin-3a by using 15% pPPG, 10% PPG, 5% cremophor eL, and 70% PBS. The 25-mM solution of nutlin-3a^{OC} was stable at room temperature for several hours; thus, this ocular formulation was used for all subsequent studies.

Subconjunctival Delivery of Nutlin-3a^{OC} Improves Intraocular Penetration

To study the intraocular penetration of nutlin-3a^{OC} we performed a pharmacokinetic study in adult C57Bl/6 mice. We administered 10 μ l of 25 mM nutlin-3a^{OC} 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 (as measured by area under the concentration-time curve; AUC) after subconjunctival administration was compared to that observed after 200 mg/kg oral (Fig. 2A) and 10 mg/kg intravenous delivery (Fig. 2B). The area under the curve (AUC) ratio of vitreous to plasma for subconjunctival administration (28.6) was approximately 2,000 fold higher than that observed after oral administration and 20,000 fold higher than intravenous administration (Fig. 2C).

Lack of Ocular and Hematologic Toxicity Associated with Nutlin-3a^{OC}

We tested nutlin-3a^{OC} for acute ocular toxicity in a cohort of 10 adult C57Bl/6 mice. A single subconjunctival injection of nutlin-3a^{OC} was administered in 1 eye. Visual acuity and intraocular pressure (IOP) were then monitored repeatedly over the next 10 days (Sup. Fig. 2A, 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-3a^{OC} or vehicle and no change in blood counts (CBC-D, data not shown). To analyze any possible hematopoietic toxicity of nutlin-3a^{OC} in greater detail, we administered 6 weekly subconjunctival injections of nutlin-3a^{OC} (10 μ L) in 15 C57Bl/6 adult mice. Three additional mice received vehicle alone. A CBC-D was performed daily for 5 days following each dose of nutlin-3a^{OC}. We found no significant differences in the white blood cell count (WBC), hemoglobin (Hb), or platelets (Plts) during the 6 weeks of testing (Sup. Fig. 2C–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). TPT has good intraocular penetration following systemic administration in murine models of retinoblastoma(6), so we tested the toxicity of nutlin-3a^{OC} combined with TPT^{syst}. We treated 4 C57Bl/6 adult mice with nutlin-3a^{OC} on Day 1 and TPT^{syst} on Days 1–5 every 3 weeks for 18 weeks (6 courses). We found no change in IOP (Sup. Fig. 2F) or CBC-D for these mice (Sup. Fig. 2G–I). We expanded the cohort to 10 C57Bl/6 mice and performed CBC-D daily for 5 days each week for 3 courses (9 weeks). There was no difference in WBC, Hb, or Plts (Sup. Fig. 2J–L).

Preclinical Testing of Nutlin3a^{OC} in Two Genetic Models of Retinoblastoma

Next we tested the efficacy of nutlin-3a^{OC}/TPT^{syst} in MDMX and p53TKO mice. If nutlin-3a blocks the MDM2/MDMX-p53 interaction in vivo, then we anticipate that nutlin-3a^{OC}/TPT^{syst} will show greater efficacy in the MDMX mice than the p53TKO mice. For this study, we incorporated alternating courses of nutlin-3a^{OC}/TPT^{syst} (Courses 2, 4, 6) with CBP^{subconj}/TPT^{syst} (Courses 1, 3, 5) (Fig. 3A). The rationale for using alternating courses is based on standard pediatric cancer clinical trial design when incorporating new agents into existing standard therapy regimens. Hereafter, this regimen will be referred to as nutlin-3a^{OC}/TPT^{syst}. We used 45 p53TKO mice and 58 MDMX mice. On Day 1 of therapy, 10 μ L CBP (10 mg/mL) was injected subconjunctivally into each eye that contained tumor, and on Days 1–5, they received TPT (0.7 mg/kg, IP). No chemotherapy was administered on Days 6–21. Before starting the second course, we performed a CBC-D to ensure recovery of all blood counts. On Day 1 of the second course of therapy, the mice received 10 μ L subconjunctival nutlin-3a^{OC} in each eye with tumor, and on Days 1–5, they received TPT^{syst} (0.7 mg/kg, IP). No therapy was administered on Days 6–21, and blood counts were monitored for recovery before initiation of Course 3. Chemotherapy was not delayed in any of the 103 animals on the study due to insufficient hematologic recovery. This alternating schedule was repeated for a total of 6 courses of therapy (18 weeks).

A total of 67 eyes were included in the analysis of the p53TKO mice, and 102 eyes were included in the analysis of the MDMX mice. CR or SD was achieved in 69% (70/102) of the MDMX eyes (Fig. 3B). In contrast, SD was achieved in only 22% (15/67) of p53TKO eyes, and CR was not achieved in any (Fig. 3B). Overall, 81% (47/58) of the MDMX mice completed 6 courses of CBP^{subconj}/TPT^{syst} nutlin-3a^{OC}/TPT^{syst} chemotherapy (Fig. 3C), and 17 unilateral enucleations were performed during treatment. The schedule of chemotherapy was not disrupted due to the enucleations. The vast majority (78%) of the p53TKO mice failed to complete therapy; 22 received bilateral enucleations. There was a significant difference ($P < 0.0001$) in the treatment responses of the 2 genetic strains. Histopathologic analysis confirmed the response to therapy, and immunoblotting showed p53-pathway activation (Fig. 3D–H). The immunoblot experiments were performed on tumors from animals that received nutlin-3a^{OC} as a single agent without TPT^{syst} to confirm molecular targeting in vivo. Thus, these results provide genetic and molecular validation that nutlin-3a^{OC} targets the p53 pathway in vivo. Representative IOP, visual acuity testing, and MRI for a MDMX mouse with complete response and a p53TKO mouse with progressive disease are shown in Fig. 4.

Preclinical Testing of Subconjunctival Nutlin-3a in a Human Orthotopic Xenograft

To complement the genetic mouse models, we developed an orthotopic xenograft model of human retinoblastoma (SJ-39) (7). This xenograft was taken directly from a patient's eye after enucleation and transplanted into the vitreous of an immunocompromised mouse's eye (Fig. 5A–D). It engrafted efficiently and was banked after the third passage in animals. The molecular, cellular, and genetic features of the xenograft are virtually indistinguishable from the primary tumor (7). Importantly, the SJ-39 xenograft, like virtually all human retinoblastomas, expresses high levels of MDMX mRNA and protein (7).

To compare the efficacy of alternating courses of subconjunctival carboplatin/systemic topotecan with subconjunctival nutlin-3a/systemic topotecan (CBP^{subconj}/TPT^{syst} _nutlin-3a^{OC}/TPT^{syst}) 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 CBP^{subconj}/TPT^{syst}, and 15 received CBP^{subconj}/TPT^{syst} _nutlin-3a^{OC}/TPT^{syst}. 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 that of the genetic models previously described, the tumors are more aggressive with shorter time to morbidity from disease than either genetic model (compare Fig. 5E to Fig. 1C). We found a slight improvement in outcome from VCR/ETO/CBP ($P = 0.0002$) (Fig. 5E) but not from CBP^{subconj}/TPT^{syst} ($P = 0.1581$) (Fig. 5F). The CBP^{subconj}/TPT^{syst} _nutlin-3a^{OC}/TPT^{syst} 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 performed on tumors from animals that received nutlin-3a^{OC} as a single agent without TPT^{syst} 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 CBP^{subconj}/TPT^{syst}. To explore targeted therapy for retinoblastoma, we developed an ocular formulation of nutlin-3a (nutlin-3a^{OC}) and

subconjunctival delivery in our preclinical models of nutlin-3a^{OC} improved intraocular penetration 2,000–20,000 fold. The combination of nutlin-3a^{OC} with TPT^{syst} 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-3a^{OC}/TPT^{syst} 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-3a^{OC}. The MDMX mice showed an overall good response to therapy with nutlin-3a^{OC}/TPT^{syst} while the p53TKO mice responded very poorly with 78% of eyes succumbing to progressive disease. These striking results confirm the direct targeting of the p53 pathway in vivo 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, 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 > 15 mm Hg 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 not a perfect correlation between tumor burden and IOP (Brennan and Dyer, unpublished) 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 two 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-3a^{OC} was when we were studying any potential hematopoietic side effects.

Following a single nutlin-3a^{OC} injection, we failed to detect any change in results from a CBC-D so we performed a follow up study with once weekly nutlin-3a^{OC} for 6 weeks. Even with this accelerated schedule, we could not detect any ocular or hematopoietic toxicity of nutlin-3a^{OC}.

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 to 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 pharmacokinetic (PK) (11, 12) and preclinical efficacy studies. Intraocular penetration of nutlin-3a following oral or i.v. administration was very poor (11, 12) so we developed an ocular formulation of nutlin-3a and tested the pharmacokinetics of nutlin-3a^{OC} following subconjunctival administration. There was a dramatic increase (2,000–20,000 increase in vitreal/plasma AUC) in intraocular penetration of nutlin-3a following subconjunctival delivery of nutlin-3a^{OC}. Toxicity studies demonstrated that subconjunctival delivery of nutlin-3a^{OC} 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-3a^{OC} was combined with TPT^{syst} in an alternating schedule with CBP^{subconj}/TPT^{syst}.

A recent study has shown that nutlin-3 can inhibit Pgp and this may in turn, increase systemic exposure of anti-cancer chemotherapeutic agents that are substrates for Pgp (17). It is unlikely that this is the primary mechanism for the anti-tumor effects observed in our MDMX genetic model of retinoblastoma or our orthotopic xenografts because pharmacokinetics 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). 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, choroid and optic nerve. This may explain why the VCR/ETO/CBP or CBP^{subconj}/TPT^{syst} 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 the great insight into the efficacy of new therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Efficacy of topotecan/carboplatin and vincristine/etoposide/carboplatin in genetic models of retinoblastoma

(A) Photograph of a p53TKO mouse retinoblastoma (arrow). (B) H&E staining of advanced-stage p53TKO retinoblastoma. (C) Step plot of age to moribund status for *Chx10-Cre;Rb^{Lox/Lox};p107^{-/-}*, *Chx10-Cre;Rb^{Lox/Lox};p107^{-/-};p53^{Lox/Lox}* (P53TKO) and *Chx10-Cre;Rb^{Lox/Lox};p107^{-/-};MDMX^{TG}* (MDMX) mice. (D) Retinal camera/fluorescein angiography images of control (C57Bl/6) and MDMX retinoblastoma at diagnosis (arrow). (E) Chemotherapy schedule for one course of therapy. (F, G) Histogram of responses of the MDMX and p53TKO model to both chemotherapeutic regimens as CR (complete response), SD (stable disease), and PD (progressive disease). (H, 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 an eyes that showed PD (K, L) and for one that showed SD (M, N). Arrows indicate viable tumor cells and open arrowhead indicated necrotic cells. Abbreviation: on, optic nerve. Scale bars: B, 0.5 mm; K, M, 25 μm; L, N, 10 μm.

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 vs. time plot was used to fit a 2-compartment model to determine the area under the curve (AUC) and calculate the ratio of the AUC in vitreous/plasma for each route of delivery.

Figure 3. Selective antitumor effects of nutlin-3a in the treatment of retinoblastoma in MDMX mice

(A) Subconjunctival carboplatin was combined with systemic topotecan for Courses 1, 3, and 5 alternating with subconjunctival nutlin-3a^{OC} combined with systemic topotecan for Courses 2, 4, and 6. (B) Quantification of response in MDMX and p53TKO model to nutlin-3a^{OC}/TPT. (C) Step plot of time to moribund status for MDMX and p53TKO mice treated with nutlin-3a^{OC}/TPT chemotherapy. (D) Immunoblot of p21 and β -actin expression from MDMX and p53TKO retinoblastoma 24 hours after nutlin-3a^{OC} administration as a single agent in vivo. (E-H) Representative H&E staining of paraffin sections from a p53 TKO eye with PD (E, F) and from an MDMX eye with CR (G, H). Arrows indicate viable cells; the open arrowhead indicates a dying cell; and the asterisks are adjacent to mitotic figures. Abbreviation: RPE, retinal pigmented epithelium. Scale bars: E, G, 25 μ m; F, H, 10 μ m.

Figure 4. Nutlin-3a^{OC}/TPT has better efficacy in MDMX mice than in p53TKO mice
(A, B) Measurements of visual acuity and intraocular pressure (IOP) in representative MDMX and p53 TKO mice treated with subconjunctival Nutlin-3a^{OC}/systemic TPT for 6 courses (18 weeks). **(C–D)** Representative T1-weighted MRI images of p53TKO **(C)** and MDMX **(D)** mice following treatment with subconjunctival nutlin-3a^{OC}/systemic TPT as indicated. Images from MRI in a sagittal **(C)** and transverse **(D)** plane through the left eye of a p53TKO mouse with PD (arrow).

Figure 5. Improved outcomes for mice with human orthotopic retinoblastoma xenografts following nutlin-3a^{OC}/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 to those receiving vincristine/etoposide/carboplatin (VCR/ETO/CBP) or subconjunctival carboplatin/topotecan (CBP/TPT) (F) or nutlin-3a^{OC}/TPT (G). (H, I) T1-weighted MRI images of an eye treated with nutlin-3a^{OC}/TPT (H) or untreated control eye. (J–M) Representative H&E staining of a paraffin section from eyes that showed progressive disease in untreated animals (J, K) and for one that showed extensive necrosis after treatment with nutlin-3a^{OC} (L, 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-3a^{OC} or vehicle treatment in vivo. Topotecan was not administered for the immunoblot studies. Abbreviations: AC, anterior chamber; C, cornea; L, lens; ON, optic nerve; Sc, sclera. Scale bars: J, L, 25 μm; K, M, 10 μm.