Original Article

Subconjunctival Carboplatin and Systemic Topotecan Treatment in Preclinical Models of Retinoblastoma

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BACKGROUND: The authors demonstrated previously that the combination of topotecan (TPT) and carboplatin (CBP) was more effective than current chemotherapeutic combinations used to treat retinoblastoma in an orthotopic xenograft model. However, systemic coadministration of these agents is not ideal, because both agents cause dose-limiting myelosuppression in children. **METHODS:** To overcome the toxicity associated with systemic TPT and CBP, the authors explored subconjunctival delivery of TPT or CBP in an orthotopic xenograft model and in a genetic mouse model of retinoblastoma (*Chx10-Cre;Rb$^{lox/lox}$;p107$^{-/-}$;p53$^{lox/lox}$*). The effects of combined subconjunctival CBP (CBP_{subcon}) and systemic TPT (TPT_{syst}) were compared with the effects of combined TPT_{subcon} and $CBP_{syst.}$ at clinically relevant dosages. **RESULTS:** Pharmacokinetic and tumor-response studies, including analyses of ocular and hematopoietic toxicity, revealed that CBP_{subcon}/TPT_{syst} was more effective and had fewer side effects than TPT_{subcon}/CBP_{syst}. **CONCLUSIONS:** For the first time, retinoblastoma was ablated and long-term vision was preserved in a mouse model by using a clinically relevant chemotherapy regimen. These results eventually may be translated into a clinical trial for children with this debilitating cancer. ***Cancer*** **2010;000:000–000.** © *2010 American Cancer Society.*

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Retinoblastoma is the third most common cancer in infants[1]; approximately 250 to 300 cases are diagnosed annually in the United States. Advances made in noninvasive focal therapies combined with chemotherapy have transformed retinoblastoma management since the 1990s. With early detection, the survival probability is approximately 90% in developed countries; in developing countries, it is only about 50%. The objective of retinoblastoma treatment is to preserve vision without compromising long-term survival while minimizing side effects. Enucleation still is common in the eye with the most advanced disease in patients who have bilateral disease.

Patients with retinoblastoma have not benefited fully from advances in drug development and local delivery methods, in part because few preclinical models faithfully recapitulate the human disease. Animal models are essential for studying retinoblastoma, because there are too few patients for large-scale clinical trials.[2] The recent development of several rodent models of retinoblastoma may facilitate advances in the treatment of bilateral retinoblastoma.[3,4] By using an orthotopic xenograft model of retinoblastoma, we demonstrated previously that the combination of topotecan (TPT) and carboplatin (CBP) delivered systemically (TPT_{syst} and CBP_{syst}) was more effective than the current standard of care (combined etoposide, vincristine, and CBP).[4] Unfortunately, coadministration of these agents causes intolerable toxicity in children.[5]

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Two approaches make possible the coadministration of these agents with minimal side effects: First, administer the drugs at different times and closely monitor blood counts to ensure that myelosuppression does not reach dangerous levels. The limitation of this approach is that tumor cells are exposed to only 1 agent at a time. Second, administer 1 drug locally to the eye and the other systemically to minimize toxicity. Abramson and colleagues were the first to demonstrate that the subconjunctival administration of CBP (CBP_{subcon}) (20 mg per eye) was a feasible treatment for retinoblastoma.[6] Indeed, retinoblastoma is ideal for local delivery of chemotherapy, because the eye is readily accessible, and high intraocular concentrations can be achieved with lower systemic exposure. Although both drugs could be administered simultaneously by subconjunctival injection, we do not favor this approach for several reasons[1]: The subconjunctival space holds a finite volume; thus, if 2 drugs are combined, then the concentration of each must be reduced.[2] Subconjunctival injections typically are performed only under anesthesia during examinations, which occur every 3 weeks. If 1 drug is delivered systemically over the course of several days, then the tumor will be exposed to that agent for a longer time.

In the current study, we compared the effectiveness of the CBP_{subcon}/TPT_{syst} combination with the effectiveness of the TPT_{subcon}/CBP_{syst} combination. Pharmacokinetics were analyzed to determine which agent was better suited to subconjunctival injection. Toxicity and tumor-response experiments also were done to guide future trials. Finally, we conducted a comprehensive preclinical study of our established knockout mouse model of retinoblastoma.[7] All diagnostic tests and assessments done in children with retinoblastoma were done in the mice.

MATERIALS AND METHODS

Cell Culture and Viability

Y79 and Weri1 cells were obtained from the American Type Culture Collection (Manassas, Va), and RB355 cells were obtained from Brenda Gallie. Retina cells were maintained in RPMI medium with 10% fetal calf serum.[8] The Y79-Luc cell line has been described previously.[4] To compare the sensitivities of retinoblastoma cell lines to different chemotherapies, we exposed each cell line to 14 concentrations (0.004-60 μM) of each drug for 0.5 hours, 2 hours, 4 hours, 8 hours, or 72 hours. The viability of each cell line was determined with the CellTiter-Glo Luminescent Assay Kit (Promega, San Luis Obispo, Calif). The luminescent signals were read by an Envision Multilabel Plate Reader (PerkinElmer, Waltham, Mass).

Genetic Mouse Model of Retinoblastoma, Orthotopic Rat Xenografts, and Fluorescent Imaging

We used the previously described *Chx10-Cre;*$Rb^{lox/lox}$*;*$p107^{-/-}$*;*$p53^{lox/lox}$ mouse model of retinoblastoma.[7] For xenograft studies, newborn Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) received an intravitreal injection of 1000 Y79-Luc cells, as described previously.[4] After approximately 2 weeks, the animals were injected intraperitoneally with D-luciferin (100 mg/kg). and image were obtained 30 minutes later using a Xenogen IVIS 200 system and Living Image Software version 2 (all from Caliper LifeSciences, Hopkinton, Mass). Tumor burden is directly proportional to the photons/cm^2 per second detected with the Xenogen imaging system.[9] Once tumor burden reached 10^6 photons/cm^2 per second, animals were used in the pharmacokinetic studies.

Pharmacokinetic Studies

Two-week-old rats were treated with TPT (10 μg per eye; GlaxoSmithKline, Research Triangle Park, NC) or CBP (100 μg per eye; Bristol-Myers Squibb, NY, NY). At serial time points (0 hours, 0.25 hours, 0.5 hours, 1.5 hours, 4 hours, and 6 hours), a cardiac puncture was performed, blood was collected, and plasma was isolated. Then, animals were killed by cervical dislocation, the eyes were removed, the vitreous was collected and flash frozen, and the retinas were harvested, rinsed in saline to remove excess drug, and flash frozen.

Total TPT (lactone plus carboxylate) was quantified by using a sensitive, specific reversed-phase, isocratic high-performance liquid chromatography.[10] The method was linear from 0.25 ng/mL to 5000 ng/mL, and the lower limit was 0.25 ng/mL. For CBP, the concentration of total platinum in supernatants was quantified using flameless atomic absorption spectrometry (Perkin Elmer AAnalyst 600 atomic absorption spectrometer with Zeeman background correction to measure platinum content) after diluting the matrix in water containing 0.2% (volume/volume) Triton X-100 and 0.06% (weight/volume) cesium chloride.

An appropriate pharmacokinetic model was fit to the TPT or CBP plasma or to the vitreous concentration-versus-time data using ADAPT software version 5.0.0

(Biomedical Simulations Resource, Los Angeles, Calif).[11] Areas under the concentration-versus-time curve (AUCs) of 0 to 6 hours for TPT or CBP plasma and for vitreous were calculated using parameter estimates and the log-linear trapezoidal method.

Intraocular Pressure Measurements

The intraocular pressure (IOP) of sedated mice was measured with the TonoLab Rebound Rodent Tonometer (TonoLab, Espoo, Finland). The device was held so that the probe was 1 mm to 4 mm from the cornea, and 6 measurements were taken and averaged. IOP measurements were taken before subconjunctival injection and then at 1 day, 2 days, and 7 days thereafter.

Visual Acuity

Visual acuity was measured using the OptoMotry System (CerebralMechanics, Inc., Lethbridge, Alberta, Canada) as described previously.[12] All tests were performed under bright-light conditions to measure cone function. At least 2 consecutive measurements were taken 24 hours before and after drug administration.

Complete Blood Counts

To assess the hematopoietic toxicity of TPT and CBP, standard complete blood counts with differential (CBC-Ds) were obtained on Day 0 and on Day 6 or Day 10 postinjection, depending on treatment. Blood ($\sim$30 μL) was collected from the facial vein and mixed with 30 μL ethanol. Samples were processed immediately using the FORCYTE Hematology Analyzer (Oxford Scientific, Oxford, Conn).

Digital Retina Camera

The initial diagnosis and staging of retinoblastoma were obtained with a Kowa retinal camera (Tokyo, Japan) that was reconfigured with a 70-diopter lens for use with mouse eyes. To observe the retina, whiskers were trimmed, and the pupil was dilated with 1% tropicamide.

Ultrasound

The Vevo 770 system (VisualSonics, Toronto, Ontario, Canada) was used for ultrasound measurements of retinoblastoma tumors. Mice were sedated with isoflurane (2%-3% in O_2) and positioned on the Vevo platform. Ultrasound gel (Aquaphora) was applied to the surface of the eye, and a 708-Hz probe was used for B-mode image acquisition.

Magnetic Resonance Imaging

Magnetic resonance images (MRIs) were obtained using a 7-T Bruker Clinscan animal MRI scanner (Bruker BioSpin MRI GmbG, Ettlingen, Germany) equipped with Bruker 12s gradient (BGA12S) and a 4-channel phase-array surface coil placed on the mouse's head. Mice were anesthetized with isoflurane (as described above) for the duration of data acquisition. Three-dimensional, magnetization-prepared rapid gradient echo (T_R, 2500 msec; T_E, 2.5 msec; T_I, 1050 ms) was used to produce T1-weighted images (0.5-mm coronal slices) with a matrix of 256 × 146 and a field of view of 30 × 20.6 mm. The initial images were read on a Siemens work station using Syngo MR B15 software (Siemens, Erlangen, Germany) and reviewed with MRIcro software (version 1.4; freeware developed by Chris Rorden; www.mricro.com).

Ocular Histopathology

Eyes were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated through an alcohol series, and washed in xylene. Then, the eyes were embedded in paraffin, and 5-μm sagittal sections were cut through the optic nerve. The corneas, ciliary epithelia, retinas, and optic nerves of untreated eyes were compared with those of treated eyes 1 day, 2 days, and 7 days after subconjunctival injection.

Statistical Methods

Significant changes in CBC-D values and blood chemistry tests were calculated with GraphPad software (GraphPad Software Inc., La Jolla, Calif) using *t* tests. Survival curves were analyzed using the Kaplan-Meier method, and a log-rank test was used to compare the curves.

RESULTS

Pharmacokinetics of Subconjunctival Injection of Carboplatin or Topotecan

Phase I clinical trials have demonstrated that CBP_{subcon}[6] or TPT_{subcon}[13] are well tolerated as single agents in patients with retinoblastoma. To determine the extent of intraocular penetration and systemic exposure of each drug after subconjunctival administration in our rodent models, we performed pharmacokinetic experiments in juvenile 2-week-old rats, as described previously.[4] TPT (10 μg per eye) and CBP (100 μg per eye) were administered as bilateral injections. On the basis of the

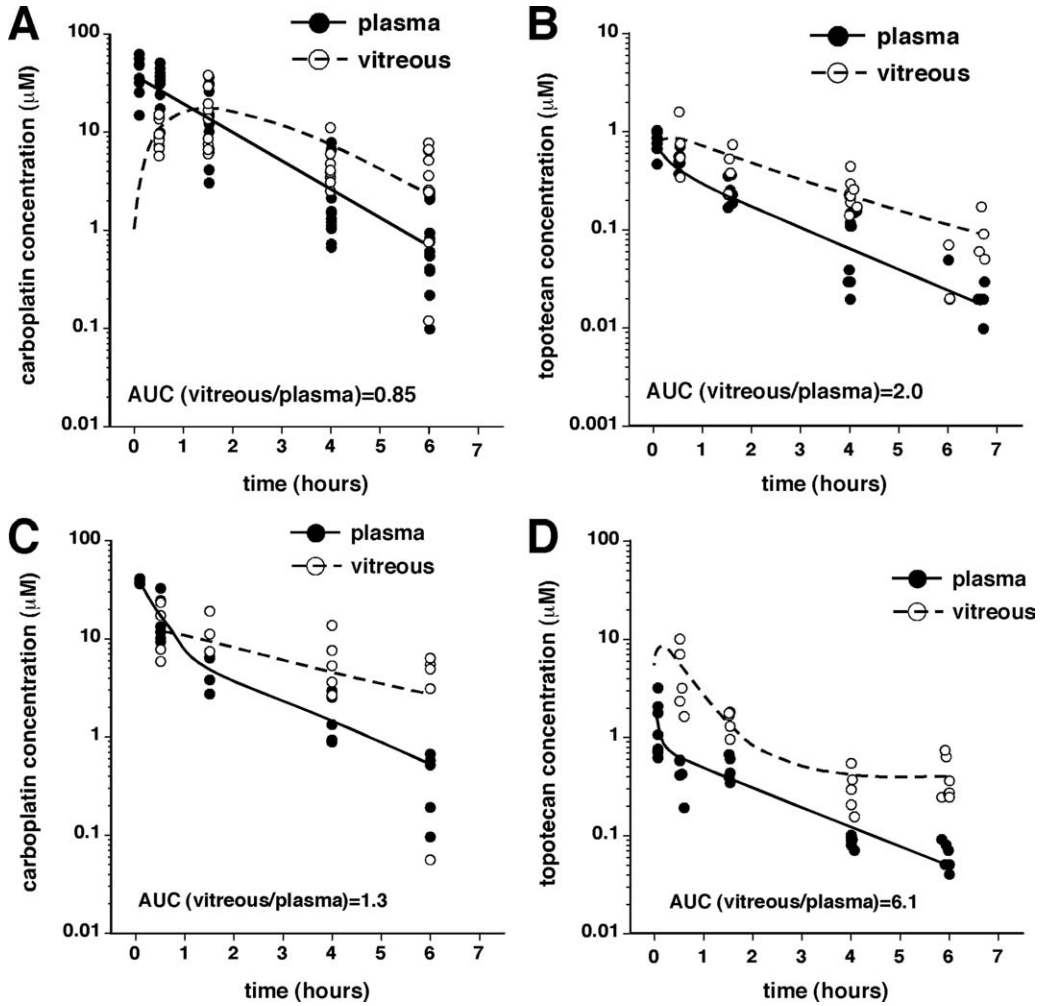

Figure 1. These graphs illustrate the pharmacokinetics of carboplatin (CBP) and topotecan (TPT) delivered individually by subconjunctival injection. (A,B) Ocular and systemic pharmacokinetics were analyzed for (A) CBP and (B) and TPT in 2-week-old rats after subconjunctival injections of either drug in both eyes (CBP, 100 μg per eye; TPT, 10 μg per eye). (C,D) In a similar experiment that was performed in tumor-bearing animals, plasma and vitreous were harvested at similar time points, and the concentration-versus-time plots were used to fit a 2-compartment model to determine the area under the concentration-versus-time curve (AUC).

proportional volume of human eyes versus rat eyes, these doses were similar to those used in children.[6,13]

The vitreous, plasma and retinas were harvested at several time points up to 6 hours after TPT_{subcon} or CBP_{subcon}, the drug concentration was measured in each tissue, and the AUCs were calculated from the model parameters. Both agents efficiently penetrated the vitreous (Fig. 1A,B; Table 1). The AUC ratio ($AUC_{vitreous}/AUC_{plasma}$) was 1.98 for TPT and 0.85 for CBP, suggesting that TPT penetrated the vitreous more efficiently.

Next, we evaluated the pharmacokinetics of TPT_{subcon} or CBP_{subcon} in tumor-bearing juvenile rats to determine whether the presence of a rapidly growing tumor in the vitreous altered the pharmacokinetic profiles of these drugs. The vitreal penetration of CBP was not altered dramatically ($AUC_{vitreous}/AUC_{plasma}$ ratio, 1.26) (Fig. 1C, Table 1). However, the vitreal penetration of TPT increased 3-fold in the presence of tumor ($AUC_{vitreous}/AUC_{plasma}$ ratio, 6.12) (Fig. 1D, Table 1). For both drugs, subconjunctival injections resulted in greater vitreous exposure than systemic injections (Table 1).[4]

To measure drug exposure in the contralateral, untreated eye after a single subconjunctival injection, we performed pharmacokinetic analyses as described above. At each time point, the $AUC_{vitreous}/AUC_{plasma}$ ratio in the

Table 1. Ocular and Systemic Topotecan and Carboplatin Exposure in Juvenile Rats

Drug (Dose)	Delivery Route	Nontumor-Bearing			Tumor-Bearing[a]		
		AUC_{Plasma} (μM/h)	$AUC_{Vitreous}$ (μM/h)	Ratio[b]	AUC_{Plasma} (μM/h)	$AUC_{Vitreous}$ (μM/h)	Ratio[b]
TPT (2 mg/kg)	Syst	2.69	1.02	0.38	NA	NA	
TPT (10 μg/eye)	Subcon	1.14	2.27	1.98	1.44	8.78	6.12
CBP (70 mg/kg)	Syst	559	330	0.59	NA	NA	
CBP (100 μg/eye)	Subcon	62.7	53.6	0.85	32.2	40.7	1.26

AUC indicates area under the concentration-versus-time curve; TPT, topotecan; Syst, systemic administration; NA, not applicable; Subcon, subconjunctival administration; CBP, carboplatin.

[a] For tumor-bearing rats, 1000 Y79-Luc cells were injected into the vitreous at on Day 0 postinjection (P0), and the animals were monitored daily from P7. When the tumor burden reached 10^6 photons/cm^2 per second, the animals were used for the pharmacokinetics study. Tumor burden was achieved by approximately P12.

[b] The $AUC_{vitreous}/AUC_{plasma}$ ratio is an estimate of the ocular exposure to each drug.

Table 2. Influence of Delivery Route on Vitreal Exposure of Topotecan and Carboplatin

Drug	$AUC_{Vitreous}/AUC_{Plasma}$ Ratio		
	Intraperitoneal[a]	Subconjunctival (Injected Eye)[b]	Subconjunctival (Contralateral Eye)[c]
Topotecan	0.38	2.0	0.35
Carboplatin	0.59	0.85	0.62

AUC indicates area under the concentration-versus-time curve.

[a] Values for intraperitoneal AUC ratios were published previously (see Laurie 2005[4]). The dose was 2 mg/kg.

[b] In independent experiments, topotecan (10 μg/eye) and carboplatin (100 μg/eye) were injected subconjunctivally into both eyes.

[c] In independent experiments, topotecan (10 μg/eye) and carboplatin (100 μg/eye) were injected subconjunctivally into the left eye, and the right eye was analyzed.

contralateral eye was lower than that in the injected eye and was similar to values reported after systemic injections (Table 2).[4]

Cytotoxicity in Retinoblastoma Cell Lines

To establish a target systemic exposure for dosing in our preclinical models, we tested the cytotoxicity of 3 human retinoblastoma cell lines (Y79, Weri1, and RB355) to TPT and CBP. The starting cell density for each line was determined empirically by measuring the growth of cells after 72 hours in 384-well culture dishes to ensure that they were within the linear range for the Promega CellTiter-Glo Assay (data not shown). The 90% inhibitory concentration (IC_{90}) in Y79 cells after TPT treatment was approximately 0.2 μM (Fig. 2A); Weri1 and Rb355 cells were more sensitive, with IC_{90} values of 0.1 μM and 0.05 μM, respectively (Fig. 2B,C). A similar trend was observed for CBP (Fig. 2A-C).

Next, we determined the duration of exposure (at 0.5 hours, 2 hours, 4 hours, 8 hours, or 72 hours) to the IC_{90} values of TPT and CBP needed to achieve maximum cytotoxicity in each line. The cells were maintained for 72 hours, and cell viability was measured (Fig. 2D-F). Ninety percent cytotoxicity was achieved in each cell line after approximately 8 hours of exposure to TPT (0.05-0.2 μM) (Fig. 2D-F), and CBP (70-200 μM) had a similar trend when we used the estimated IC_{90} values from the AUCs in Figure 2 (see Fig. 2A-C). Although these values were not equivalent to AUCs, they approximated the minimal sustained levels of CBP or TPT required to kill a significant proportion of retinoblastoma cells in the vitreous.

The AUCs for vitreal exposure ($AUC_{vitreous}$) for each agent using each route of administration were used to determine a ratio of the 2 drugs that would be achieved with each approach. The $AUC_{vitreous}$ for TPT_{syst} (2 mg/kg) was 1.02 μM per hour, which was equivalent to 0.102 μM per hour for the dose of 0.2 mg/kg used in our animal studies. The $AUC_{vitreous}$ for CBP_{subcon} (100 μg per eye) was 53.6 μM per hour. Therefore, the ratio in the vitreous when CBP_{subcon}/TPT_{syst} was administered was 0.1 μM TPT/53 μM CBP; when the methods of delivery were reversed, the ratio was 2.27 μM TPT/165 μM CBP. Clearly, these concentrations were not achieved in the vitreous (Fig. 1), but the overall exposure ratio was reflected

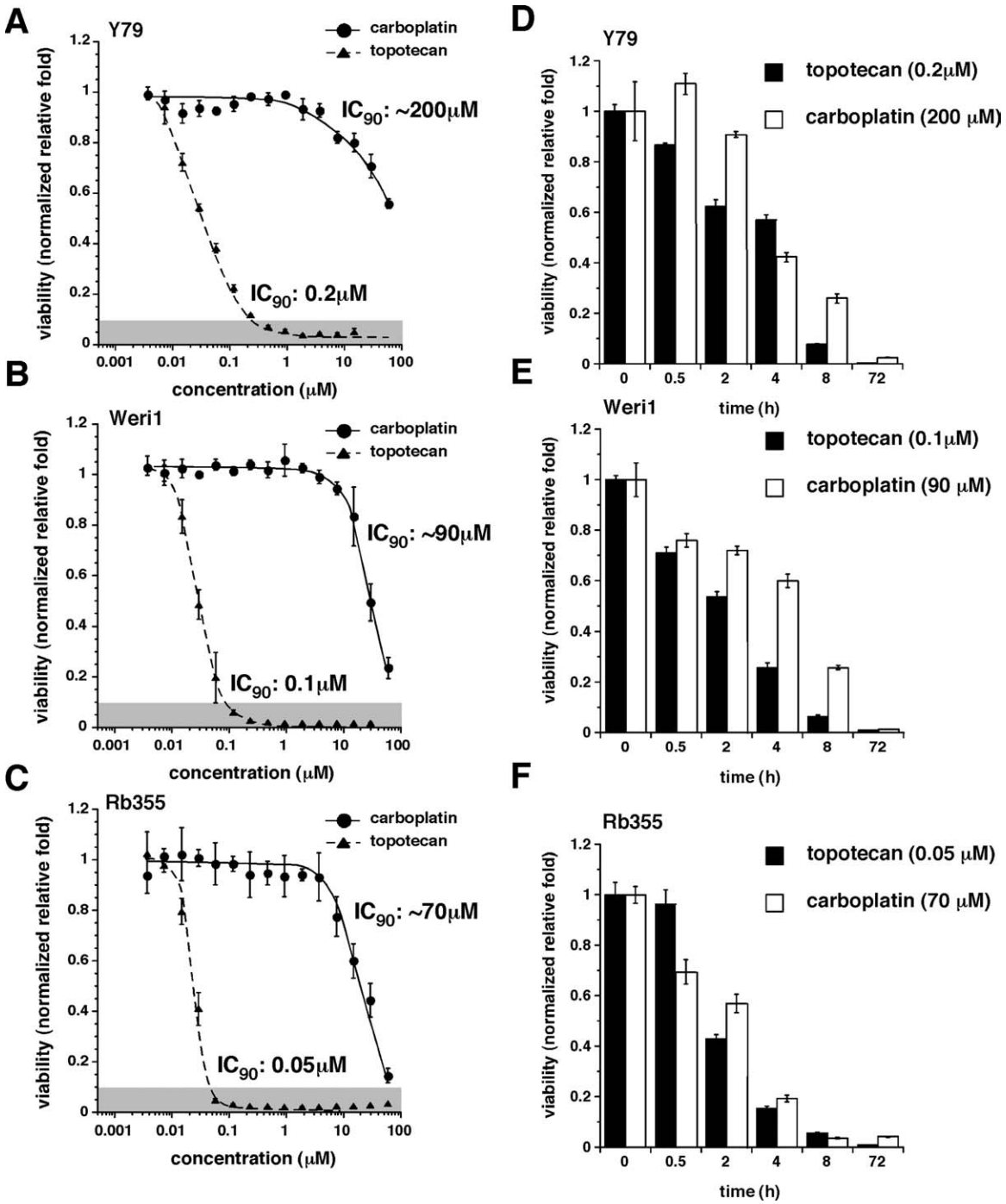

Figure 2. The sensitivity of retinoblastoma cell lines to topotecan (TPT) and carboplatin (CBP) is illustrated. (A-C) The viability of retinoblastoma cells from the cell lines (A) Y79, (B) Weri1, and (C) RB355 cells was observed after 72 hours of exposure to different concentrations of CBP or TPT. Each data point represents the mean ± standard deviation of triplicate samples. (D-F) These charts illustrate the viability of retinoblastoma cells exposed at the 90% inhibitory concentration (IC_{90}) of each drug for different periods. The drug was washed off, and cells were incubated for 72 hours as described for A, B, and C. Each vertical line represents the mean ± standard deviation of triplicate samples.

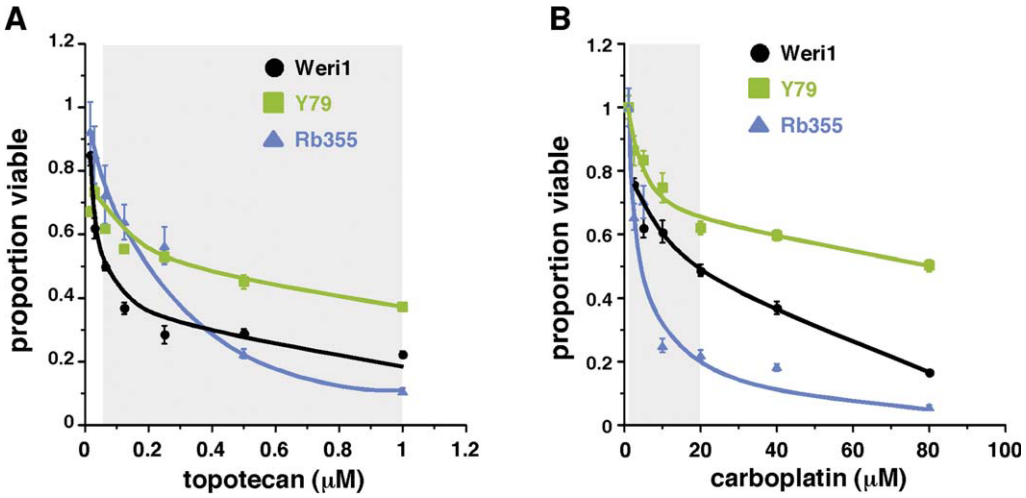

Figure 3. The sensitivity of retinoblastoma cells to combination chemotherapy is illustrated. Cells from the retinoblastoma cell lines Y79, Weri1, and RB355 were exposed to different dilutions of fixed ratios of carboplatin (CBP)/topotecan (TPT) based on pharmacokinetic data for vitreal exposure of the 2 drugs after subconjunctival and systemic administration. (A) Data are plotted for dilutions of the 2 drugs at the ratio achieved in the vitreous when the combination of subconjunctival CBP and systemic TPT was administered. The dose of TPT is plotted for each dilution. Each data point is the mean ± standard deviation of triplicate experiments. (B) Data are plotted for dilutions of the 2 drugs at the ratio achieved in the vitreous when the reverse combination was administered. The dose of CBP is plotted for each dilution. Each data point is the mean ± standard deviation of triplicate experiments. The gray box represents the range of drug concentration in the vitreous from pharmacokinetic experiments.

in these numbers. To determine whether 1 ratio was more effective than the other and to guide our tumor-response experiments, we performed a dose-response analysis using these 2 ratios. Each ratio had substantial toxicity across the concentration ranges achieved in the vitreous (Fig. 3).

Tumor Response to Subconjunctival Injection of Topotecan or Carboplatin

To test whether TPT_{subcon}/CBP_{syst} and CBP_{subcon}/TPT_{syst} elicited different tumor responses, we performed a tumor-response experiment using our rat xenograft model. The animals were divided randomly divided into 3 groups: saline, TPT_{subcon} (10 μg per eye)/CBP_{syst} (10 mg/kg), and CBP_{subcon} (100 μg per eye)/TPT_{syst} (0.2 mg/kg daily for 5 days). The drugs doses used in this study recapitulates those used in clinical trials as closely as possible, taking into account species-specific toxicity (calculations available upon request). In the saline-treated group, the tumor burden increased by approximately 50-fold to 100-fold (Fig. 4A); and, for the treated groups, it decreased by approximately 10-fold ($P < .01$) compared with the saline-injected group after 7 days (Fig. 4B,C). Examples of an untreated rat and a treated rat (CBP_{subcon}/TPT_{syst}) with corresponding histopathology are provided in Figure 4D. One of the most striking and surprising differences between the 2 groups was the morbidity associated with TPT_{subcon}/CBP_{syst} (Fig. 4C). In the group that received this combination, no animals survived past Day 5 of chemotherapy.

Ocular Toxicity After Subconjunctival Injection of Topotecan or Carboplatin

We randomly assigned 12 C57Bl/6 mice into 3 groups: untreated, $saline_{subcon}$ (10 μL per eye), unilateral TPT_{subcon} (10 μg per eye), and unilateral CBP_{subcon} (100 μg per eye) and assessed ocular toxicity (ie, inflammation and other periocular side effects) 1 day, 3 days, and 7 days thereafter. No ocular toxicity was associated with any injection (Fig. 5A). We also monitored the animals for elevated IOP and impaired visual acuity but observed no evidence of change in either measure at any time point (Fig. 5B,C).

Then, we combined subconjunctival injections with systemic administration to determine whether ocular toxicity was caused by exposure of eye structures to the agents. By using 3 C57Bl/6 mice per group in 2 groups, we compared the ocular toxicity, visual acuity, and IOP in animals that received TPT_{subcon} (10 μg per eye)/CBP_{syst} (18 mg/kg) or CBP_{subcon} (100 μg per eye)/TPT_{syst} (0.1 mg/kg). After 1 day, 3 days, and 7 days, we observed no difference in any measure for any group (data not shown). Histopathologic analysis confirmed that no obvious changes occurred in the retinas, ciliary epithelia, or

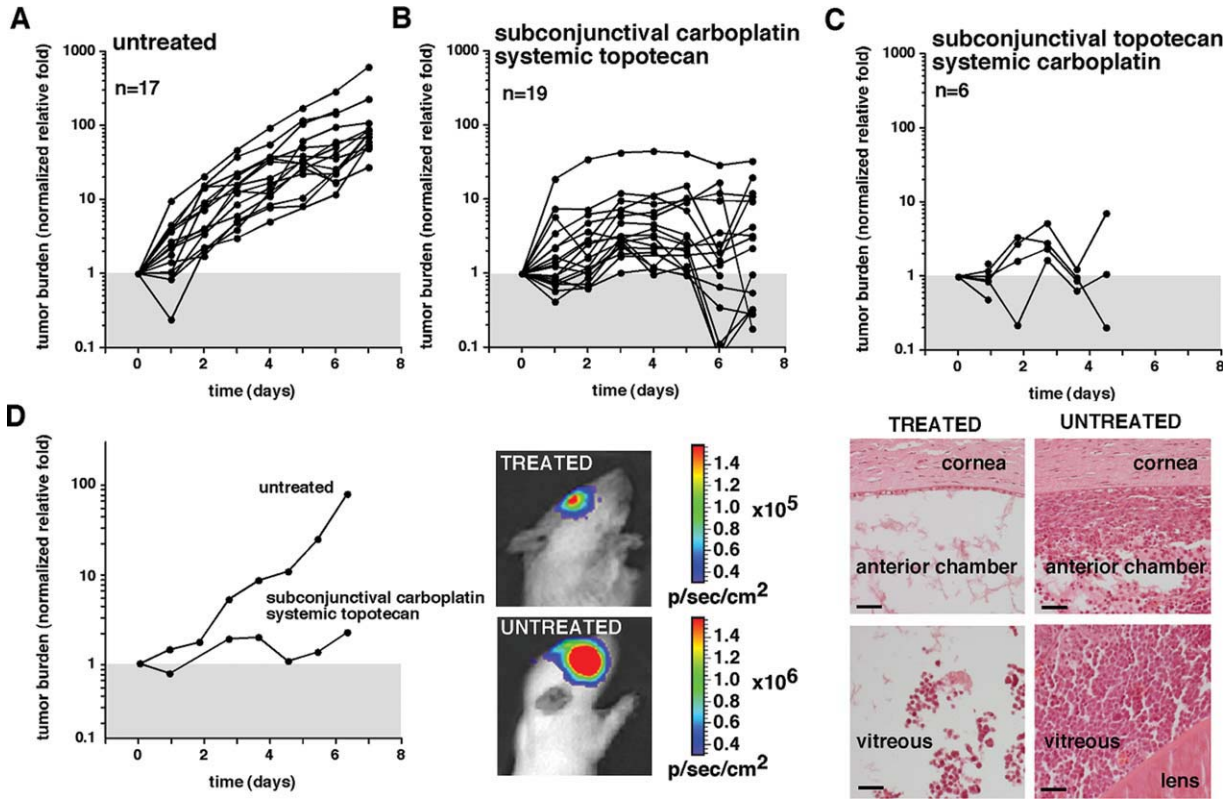

Figure 4. Tumor response to subconjunctival topotecan (TPT_{subcon}) combined with systemic carboplatin (CBP_{syst}) and to CBP_{subcon} combined with TPT_{syst} is illustrated. (A-C) In rats with orthotopic xenografts, (A) the first group received saline injections, (B) the second group received CBP_{subcon} (100 μg per eye)/TPT_{syst} (0.2 mg/kg daily for 5 days), and (C) the third group received TPT_{subcon} (10 μg per eye)/CBP_{syst} (18 mg/kg). All data were normalized to the starting tumor burden to provide relative growth and response. (D) Bioluminescence measurements and histopathologic analyses of an untreated animal and an animal that received CBP_{subcon}/TPT_{syst} are shown (p/sec/cm^2 indicates photons per second per cm^2). Scale bars = 25 μm.

corneas of eyes that were exposed to CBP_{subcon} or TPT_{subcon} (data not shown).

Myelosuppression and Dehydration Associated With Subconjunctival Topotecan and Systemic Carboplatin

Next, we examined drug-induced, systemic toxicity. By using seven 8-day-old rats, we administered CBP_{subcon} (100 μg)/TPT_{syst} (0.2 mg/kg daily for 5 days) to mimic the TPT dose administered to children with retinoblastoma. Body weights were measured daily for 9 days and compared with those of untreated littermates; no significant weight loss was detected (Fig. 6A). Blood samples drawn on Days 0 and 10 revealed no reduction in CBC-D measures after treatment (data not shown).

In a similar set of experiments with the delivery of agents reversed and at clinically relevant doses (ie, TPT_{subcon} 10 μg per eye/CBP_{syst} 34 mg/kg), the juvenile rats could not tolerate the treatment (data not shown). When we reduced the CBP dose to 10 mg/kg, 3 of 6 animals survived to Day 6 of treatment but exhibited signs of profound dehydration (ie, significant weight loss, lethargy, and tenting of the skin; data not shown) (Fig. 6A). Blood chemistries obtained from the surviving rats on Day 6 were normal except for an elevated blood urea nitrogen level, consistent with chemotherapy-related dehydration (Fig. 6B). In addition, CBC-D measures were consistent with myelosuppression, as evidenced by neutropenia and thrombocytopenia (Fig. 6C). These data indicate that TPT_{subcon}/CBP_{syst} is significantly more toxic than the reverse treatment delivery in juvenile rats.

Longitudinal Study of Systemic Topotecan and Subconjunctival Carboplatin in a Preclinical Model of Retinoblastoma

The orthotopic xenograft model is useful for short-term pilot studies; however, for long-term studies, the genetic mouse model is preferred because it better recapitulates

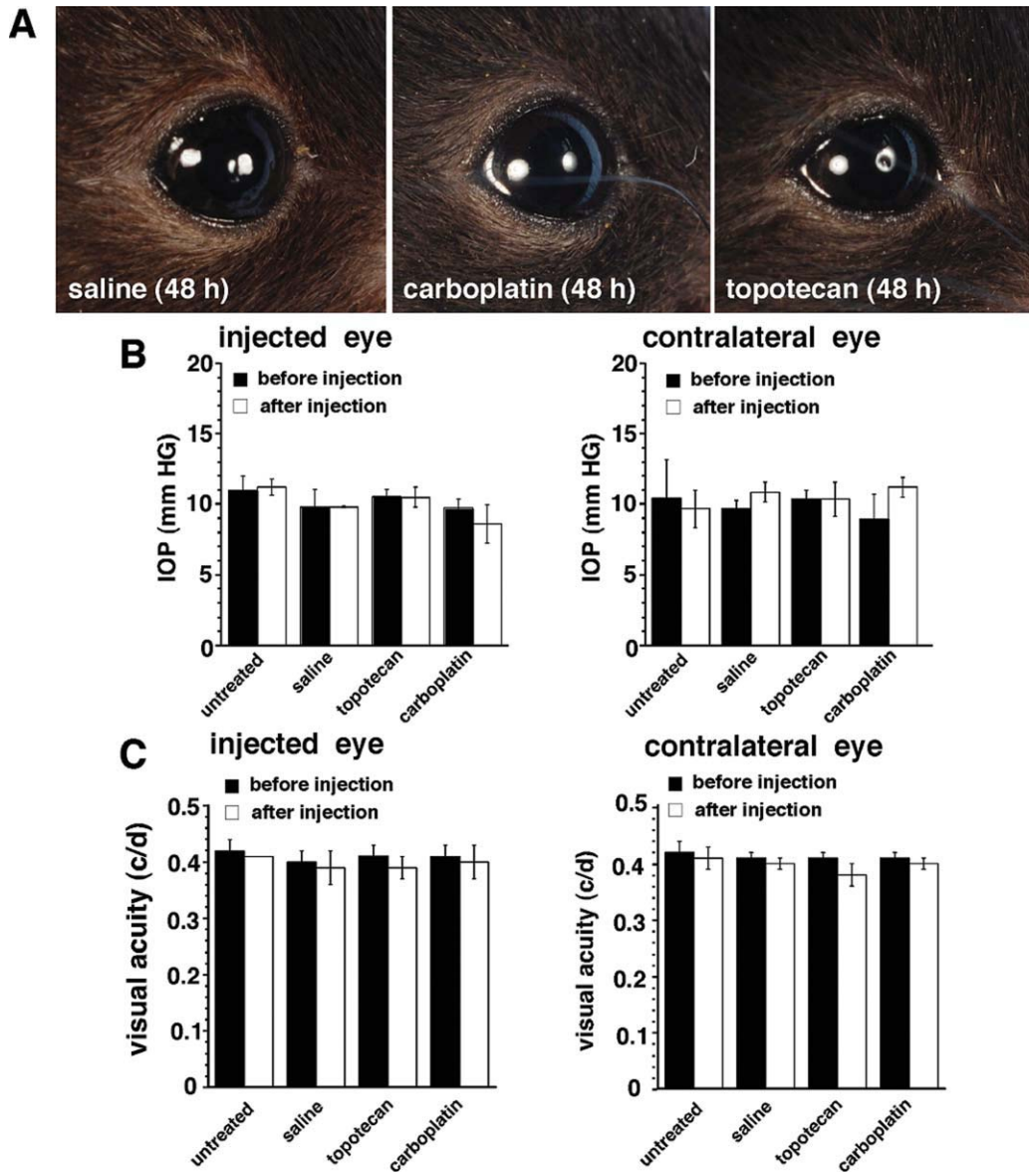

Figure 5. The ocular effects of subconjunctival administration of chemotherapy were analyzed. (A) These are photographs of the eyes of C57Bl/6 mice 48 hours after subconjunctival injections of 10 μL saline, 100 μg carboplatin, or 10 μg topotecan. (B) Intraocular pressure (IOP) was measured before and after the administration of either drug. Each bar represents the mean ± standard deviation of 6 measurements from 3 animals. (C) Changes in visual acuity as a result of subconjunctival chemotherapy injections were measured before and after injection in groups of 3 animals per treatment; the contralateral eye was used as a control. Data represent the mean ± standard deviation of 2 measurements from 3 animals in each group.

the human disease.[7] To determine whether mice can tolerate CBP_{subcon}/TPT_{syst} at a clinically relevant dose and whether this combination alters tumor progression, we performed a preclinical trial using *Chx10-Cre; $Rb^{Lox/Lox}$;$p107^{-/-}$;$p53^{Lox/Lox}$* mice (Fig. 7A).[7] If they were left untreated, 95% (122 of 129 mice) developed retinoblastoma, and 79% (97 of 122 mice) developed bilateral disease (Fig. 7A).

Starting at age 6 weeks, the mice were screened for retinoblastoma (Fig. 7A). Once a tumor was detected (Fig. 7B), baseline measurements were established for CBC-D, visual acuity, and IOP. Then, each animal received six 3-week courses of chemotherapy (Fig. 7A) to recapitulate current clinical protocols for retinoblastoma. On Day 1, each animal received CBP_{subcon} (100 μg) in the affected eye(s); on Days 1 through 5, each animal

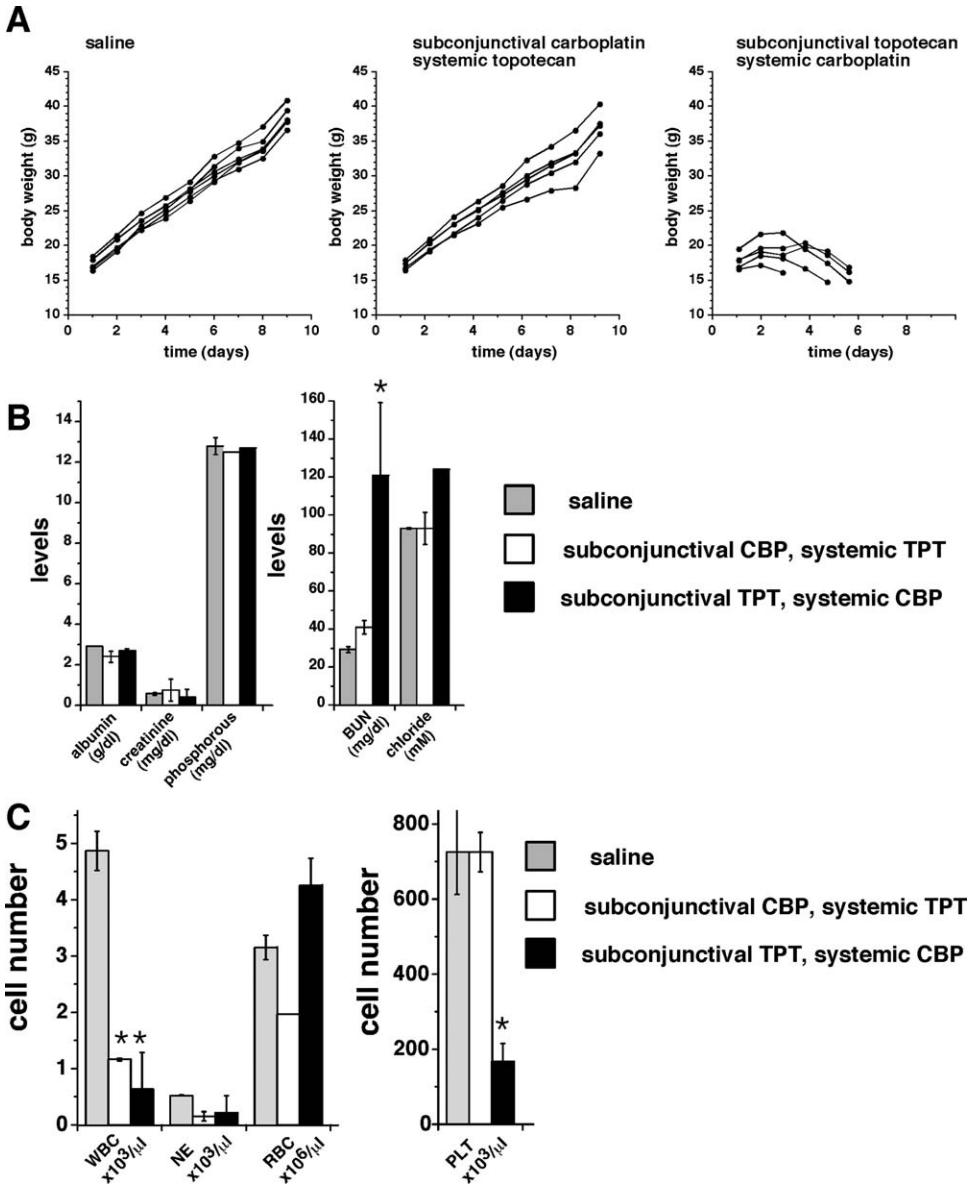

Figure 6. Side effects of topotecan (TPT) and carboplatin (CBP) combination chemotherapy are illustrated using different routes of administration. (A) The first group received saline injections, the second group received subconjunctival CBP (CBP_{subcon}) (100 μg per eye)/systemic TPT (TPT_{syst}) (0.2 mg/kg daily for 5 days), and the third group received TPT_{subcon} (10 μg per eye)/CBP_{syst} (10 mg/kg daily for 5 days). Body weights were measured each day for the subsequent 9 days. When TPT_{subcon}/CBP_{syst} was administered, all animals died with signs of dehydration by Day 6. (B) Blood chemistry results from treated and untreated juvenile rats are shown. Each bar represents the mean ± standard deviation of measures from 2 or 3 animals. BUN indicates blood urea nitrogen. (C) Complete blood counts with differential are illustrated from treated and untreated juvenile rats. Each bar represents the mean ± standard deviation based on data from 2 or 3 animals. Asterisks indicate statistical significance with $P < .05$. WBC indicates white blood cells; NE, neutrophils; RBC, erythrocytes; PLT, platelets.

received TPT_{syst} (0.1 mg/kg). The animals then had 2 weeks off therapy to complete the 3-week course. Tumors were monitored by digital retinal camera, ultrasound, and MRI (Fig. 7B); and IOP, visual acuity, and CBC-D were measured. If the tumor progressed during treatment in 1 eye but the other eye was favorable, then surgical enucleation was performed. The animal then continued on the study according to the predetermined schedule. Of the 42 eyes from 22 animals in this study, 2 had a complete response, 11 had stable disease, and 29 had disease progression (Fig. 7C, Table 3). The period required for 50% of the animals to reach moribund status, which was

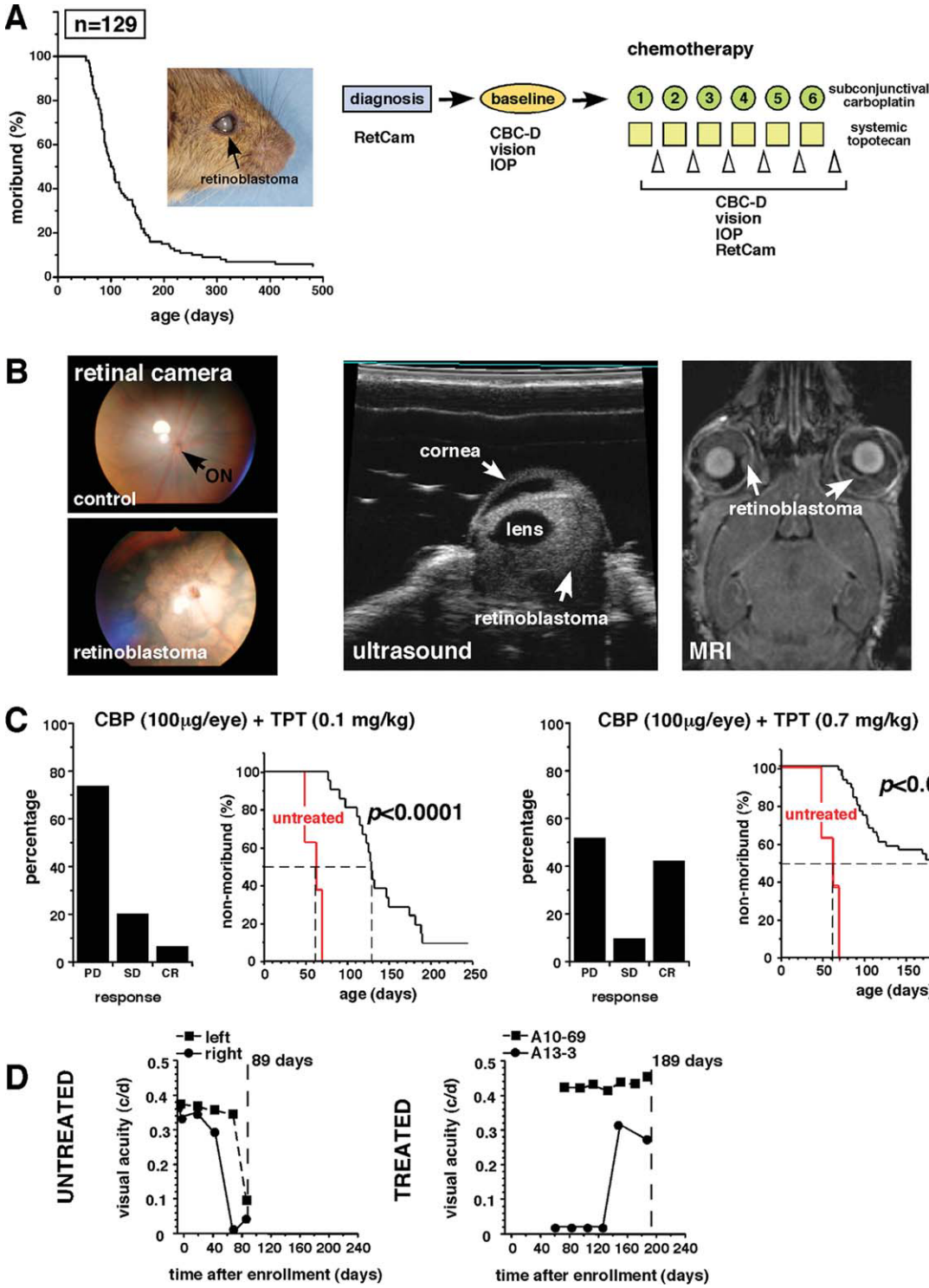

Figure 7. These charts illustrate the longitudinal study of subconjunctival carboplatin (CBP_{subcon}) and systemic topotecan (TPT_{syst}) administration in a genetic model of retinoblastoma. (A) In a genetic mouse model of retinoblastoma *Chx10-Cre;*$Rb^{lox/lox}$*;*$p107^{-/-}$*;*$p53^{lox/lox}$, mice developed aggressive and invasive retinoblastoma with 90% moribundity by age 250 days. Starting at age 6 weeks, the animals were screened for tumors. Once the baseline tumor was established, their chemotherapy trial was initiated. Each course included a single dose of CBP_{subcon} (100 μg per eye) on Day 1 and TPT_{syst} (0.1 mg/kg) on Days 1 through 5 of the 21-day course. Around Day 20 or 21, complete blood counts with differential (CBC-D), visual acuity, and intraoccular pressure (IOP) were measured, and retinal images were acquired. RetCam indicates retinal camera. (B) These are representative images of an eye without detectable retinoblastoma and from an untreated eye with retinoblastoma before to the study. If left untreated, then tumors filled the vitreous, as observed on ultrasound and on a magnetic resonance image (MRI). (C) These are histograms of tumor response after 6 courses of CBP_{subcon}/TPT_{syst} in which (*Left*) 0.1 mg/kg TPT was administered. (*Right*) When this combination included the clinically relevant area under the concentration-versus-time curve (AUC)-guided dose of TPT (0.7 mg/kg), the response improved. The time to moribund status for treated animals was significantly longer ($P<.0001$) than that for the 8 untreated animals that were monitored in parallel. (D) Visual acuity (in cycles per degree [c/d]) is illustrated in an untreated animal that rapidly worsened as the tumor progressed. In treated eyes from 2 independent readings, vision was preserved for at least 189 days in 1 animal (A10-69) and was restored after 130 days in another animal (A13-3).

Table 3. Preclinical Testing of Subconjunctival Carboplatin/Systemic Topotecan in a Genetic Model of Retinoblastoma

Animal No.	Eye	Age, wk	Disease Stage	Outcome	Notes
A10-62	Right	9	3	SD	Normal visual acuity, IOP, CBC-D
A10-62	Left	9	3	SD	Normal visual acuity, IOP, CBC-D
A10-46	Right	9	4	PD	Elevated IOP
A10-46	Left	9	2	PD	Reduced visual acuity
A8-17	Right	8	3	PD	Enucleation
A8-17	Left	8	3	PD	Enucleation
A8-15	Right	9	3	PD	Enucleation
A8-15	Left	9	3	PD	Enucleation
A9-23	Right	7	3	PR	Normal visual acuity, IOP, CBC-D
A9-23	Left	7	3	PR	Normal visual acuity, IOP, CBC-D
A8-19	Right	6	3	PD	Reduced visual acuity
A8-19	Left	6	3	PD	Reduced visual acuity
A9-38	Right	9	0	PD	Elevated IOP, loss of vision
A9-38	Left	9	2	PD	Elevated IOP, loss of vision
A9-37	Right	8	3	PD	Elevated IOP, loss of vision
A9-37	Left	8	3	PD	Elevated IOP
A10-65	Right	7	3	PD	Enucleation
A10-65	Left	7	3	SD	Reduced visual acuity
A10-63	Right	7	3	PD	Enucleation
A10-63	Left	7	2	PR	Normal visual acuity, IOP, CBC-C
A10-69	Right	9	0		No tumor
A10-69	Left	9	3	CR	Normal visual acuity, IOP, CBC-D
A13-5	Right	9	3	PD	Elevated IOP, loss of vision
A13-5	Left	9	2	PD	Elevated IOP, loss of vision
A10-67	Right	7	3	SD	Normal IOP
A10-67	Left	7	3	PD	Enucleation
A13-3	Right	9	3	SD	Normal IOP
A13-3	Left	9	3	CR	Restoration of vision
A13-1	Right	9	3	PD	Elevated IOP, loss of vision
A13-1	Left	9	2	SD	Normal IOP
A11-75	Right	6	0		No tumor
A11-75	Left	6	2	SD	Normal visual acuity, IOP
A8-11	Right	8	3	PD	Normal IOP, loss of vision
A8-11	Left	8	2	PD	Elevated IOP, loss of vision
A8-13	Right	8	2	CR	Normal visual acuity, IOP, CBC-D
A8-13	Left	8	0	PD	Normal visual acuity, IOP, CBC-D
A9-31	Right	8	2	PD	Enucleation
A9-31	Left	8	3	PD	Enucleation
A8-3	Right	8	0	PD	Enucleation
A8-3	Left	8	3	PD	Enucleation
A8-5	Right	8	3	PD	Enucleation
A8-5	Left	8	3	PD	Enucleation
A9-25	Right	8	3	PD	Enucleation
A9-25	Left	8	4	PD	Enucleation

SD indicates stable disease; IOP, intraocular pressure; CBC-D, complete blood count with differential; PD, progressive disease; PR, partial response; CR, complete response; SD, stable disease.

defined by imminent ocular rupture because of tumor filling the eye, increased from 60 days in untreated mice to 125 days in treated mice (Fig. 7C).

Next, we tested the higher, clinically relevant, AUC-guided dose of 0.7 mg/kg TPT_{syst} on the same schedule in combination with CBP_{subcon}. We treated 44 animals (80 eyes) for 4 courses. To date, 43% (19 of 44 animals) had a complete response and are long-term survivors (>270 days) (Fig. 7C). Both doses of TPT were associated with a significant improvement in outcome ($P < .0001$). Remarkably, vision was restored or preserved in 73% of the animals that had a complete response after CBP_{subcon}/TPT_{syst} (Fig. 7D).

DISCUSSION

Retinoblastoma is unique among pediatric solid tumors, because locally delivered and systemically administered chemotherapy can be combined to optimize intraocular drug exposure while minimizing the side effects associated

with combination chemotherapy. We tested the feasibility, efficacy, and toxicity associated with this approach and observed that the CBP_{subcon}/TPT_{syst} combination resulted in greater efficacy and fewer side effects in juvenile rats with orthotopic xenografts. No ocular side effects were detected after acute exposure or repeated dosing on a clinically relevant schedule. Then, these findings were validated in a longitudinal study of six 3-week courses administered to a knockout mouse model of retinoblastoma. For the first time to our knowledge, we ablated retinoblastoma in mice, and vision was restored in some long-term survivors. Although these data are promising for stopping retinoblastoma in vivo in a genetic model of retinoblastoma, we still do not know whether it will provide any predictive power for improved outcome in human retinoblastoma. Pharmacokinetic studies are essential for determining the vitreal exposure and the relative plasma exposure for a given dose. This is particularly important for the TPT/CBP combination; because, if the systemic exposure of both drugs is too high, then dose-limiting myelosuppression or other toxicities will develop. Our pharmacokinetic studies resulted in several key findings: 1) Subconjunctival delivery of either agent efficiently penetrated the eye, as indicated by the vitreal concentration of drugs; however, 2) the $AUC_{vitreous}/AUC_{plasma}$ ratios indicated that the intraocular penetration of TPT was better than that of CBP.[3] The presence of tumor in the eye slightly increased the penetration of both drugs.[4] Subconjunctival administration led to greater vitreal exposure than systemic administration of either drug.[5] After unilateral subconjunctival injection, the contralateral eye revealed detectable vitreal exposure to the drug as a result of its uptake into the circulation. These data indicate that subconjunctival delivery of either drug is feasible for the treatment of retinoblastoma. Visual acuity, IOP, and cytotoxicity analyses revealed no detectable ocular toxicity associated with subconjunctival injection of either drug. In addition, when combined with systemic exposure to the other drug, no changes in ocular physiology or histology were observed.

In contrast, chemotherapy-related dehydration and myelosuppression were major challenges in these studies when a clinically relevant dose of TPT_{subcon}/CBP_{syst} (TPT, 10 μg per eye; CBP, 34 mg/kg) was administered. Even when the CBP dose was reduced to 10 mg/kg, the animals developed signs of severe dehydration and myelosuppression. This was surprising, because no detectable toxicity or side effects were observed when the delivery methods were reversed despite similar tumor response. We speculate that this was caused by the increased overall exposure of the 2 agents with this route of delivery because of the large dose of CBP used for systemic administration. The important advantage of the CBP_{subcon}/TPT_{syst} combination is that TPT can be delivered on the "daily for 5 days" schedule that is used clinically. This approach provides continued chemotherapeutic exposure for several days, which is not possible when the methods of delivery are reversed, because TPT_{subcon} is administered only on Day 1 of therapy.

Toxicity data from our orthotopic xenograft model confirmed that the preferred drug delivery is CBP_{subcon}/TPT_{syst}. Tumor response to TPT_{subcon}/CBP_{syst} beyond 5 days could not be monitored in the rats because of morbidity. The advantage of this model is that it is well characterized and standardized, and direct comparisons can be made with previous studies of retinoblastoma[4]; the disadvantage is that only short-term studies can be conducted because the tumors grow quickly. Thus, we combined preliminary studies in this model with long-term studies in our genetic mouse model.

We validated the feasibility of multiple 3-week courses of CBP_{subcon}/TPT_{syst} to treat retinoblastoma by using the *Chx10-Cre;*$Rb^{Lox/Lox}$*;p107*$^{-/-}$*;*$p53^{Lox/Lox}$ mouse model. The animals received a comparable dose on the same schedule used to treat patients with retinoblastoma. CBC-D measures were closely monitored, and the mice recovered well on the treatment regimen. Tumor response was observed in a substantial proportion of the animals, as measured by reduced tumor burden, recovery of vision, maintenance of normal IOP, and long-term survival (up to 1 year). At a subclinical dose of TPT (0.1 mg/kg), treated animals fared better than untreated animals; however, long-term survival and restoration of vision were achieved only at the clinically relevant, AUC-guided dose (0.7 mg/kg). Periocular CBP can cause significant scar tissue in children with retinoblastoma. In our studies, we did not observe any scar tissue in the mice; however, this remains a significant challenge for subconjunctival delivery in patients. It may be possible to develop an alternative delivery device to direct drug across the sclera without exposure to the subconjunctival tissue.

One important difference between our study and the clinical treatment of retinoblastoma is that children with retinoblastoma also receive focal therapies, such as laser treatment. We propose that mice can tolerate CBP_{subcon} ($\leq$20 mg per eye) combined with TPT_{syst} (0.7 mg/kg) for 6 3-week courses using doses that are comparable to those used previously to treat patients with retinoblastoma. More

important, these data establish the feasibility of conducting preclinical drug studies in genetic and orthotopic xenograft animal models of retinoblastoma.

CONFLICT OF INTEREST DISCLOSURES

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