Subconjunctival Topotecan in Fibrin Sealant in the Treatment of Transgenic Murine Retinoblastoma

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PURPOSE. To test the effects of subconjunctival topotecan (TPT) in fibrin sealant (FS) in transgenic murine retinoblastoma (RB).

METHODS. Growth inhibitory, apoptotic, and cell cycle effects of TPT were assayed in human RB cell lines. In a dose-escalation study, eight groups of three 10- to 14-week-old wild-type mice were treated bilaterally with a single 30-μL injection of subconjunctival TPT in FS (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, or 3.2 mg/mL). Two groups of twenty 10-week-old LHβ-Tag transgenic mice were then treated in the right eye only with TPT in FS (3.2 mg/mL in 30 μL; 0.1-mg total dose) or with FS only. The contralateral eye in each group was left untreated to serve as an internal control. After 3 weeks, ocular tumor burden was determined by histologic examination.

RESULTS. At 48 hours, I$_{50}$ values of TPT in Y79 and Weri-Rb1 RB cell lines were 35 nM and 50 nM, respectively. Growth inhibitory effects were correlated with increased apoptosis and accumulation of cells in G2. Cytotoxicity of TPT was comparable in aqueous media and in FS. In the dose-escalation study, no histopathologic evidence of ocular toxicity was observed at any dose. Clinical toxicities (mild enophthalmos and eyelid alopecia) were observed only at the highest dose tested (3.2 mg/mL). In the treatment study, both eyes of TPT-treated mice demonstrated significant reduction in tumor burden compared with both eyes of mice treated with FS only (59% reduction; P = 0.04). In mice treated with TPT, tumor burden in TPT-treated eyes and in untreated contralateral eyes did not differ significantly.

CONCLUSIONS. Subconjunctival administration of TPT in FS to one eye allows the formation of a TPT depot sufficient for an effect to occur 3 weeks after treatment. This effect—bilateral reduction in tumor burden without a significant difference in treated versus untreated eyes—suggests that the major route of drug delivery in this system is hematogenous rather than transscleral. (Invest Ophthalmol Vis Sci. 2008;49:490–496) DOI:10.1167/iovs.07-0653

Retinoblastoma (RB) is a pediatric retinal tumor caused by mutation in both alleles of the retinoblastoma tumor-suppressor gene (RB1).1,2 Radiotherapy is frequently curative but increases the already substantial risk for second primary tumors in patients with heritable disease.3 Consequently, most specialists use systemic combination chemotherapy (usually carboplatin, etoposide, and vincristine) with focal therapy (laser or cryotherapy) as first-line treatment. Advanced disease with vitreous seeding is less responsive to combination chemotherapy, presumably because intravenous delivery does not reliably produce therapeutic intravitreal drug levels.

Higher levels of carboplatin can be achieved in the retina and vitreous by periocular administration in aqueous vehicle.4 This approach relies on transscleral diffusion to deliver drug locally. Drug injected into the periocular space can also reach the eye through the systemic circulation on clearance by the conjunctival lymphatics and blood vasculature.5–7 For reasons that remain largely unexplored, agents are cleared by local vasculature with varying efficiency. Some drugs persist in the periocular region, reaching the eye predominantly transsclerally after local injection.8,9 Others are eliminated before significant transscleral diffusion can occur, reaching the eye primarily through the hematogenous route.5,6 Higher intraocular levels of such agents can, nevertheless, be achieved by administration of a dose large enough to saturate vascular clearance mechanisms, thereby creating a drug depot for transscleral diffusion.6

Retinoblastoma specialists have reported promising therapeutic results using periocular carboplatin in aqueous vehicle.10,11 In 2007, the National Cancer Institute Children’s Oncology Group opened a Phase III clinical trial that includes periocular carboplatin for patients with international stage C and D disease. However, this modality has been associated with serious adverse effects, including optic atrophy,10 ocular motility changes, and orbital fat necrosis with fibrosis,11 severe pseudopapetal cellulitis,12 and ischemic necrosis with atrophy of the optic nerve and subsequent blindness.13–15 These toxicities likely occur because of rapid dispersal of the entire delivered dose of carboplatin throughout the periocular space and consequent damage to extraocular tissues.

Fibrin sealant (FS) is a Food and Drug Administration-approved biodegradable surgical adhesive that could be used as a controlled-release vehicle for periocular drug delivery. By increasing intraocular drug exposure and reducing periocular drug levels, FS could provide more effective tumor control while reducing orbital toxicities associated with aqueous vehicle. Studies in rabbits have demonstrated that subconjunctival carboplatin in FS provides 2-fold higher peak intravitreal drug levels than systemic chemotherapy and sustained intraocular...
drug levels for up to 2 weeks with minimal toxicity.\textsuperscript{14,15} We recently reported successful local treatment of \textit{LH\textbeta}-\textit{Tag} transgenic murine RB with a single subconjunctival injection of carboplatin in FS (91\% reduction in tumor burden versus vehicle-treated controls, \textit{P} < 0.004).\textsuperscript{16} Based on these data, we have opened a phase 1 trial of sub-Tenon carboplatin in FS for eyes that would otherwise require enucleation.

Topotecan (TPT) is a topoisomerase \textit{I} inhibitor that has demonstrated early promise as a therapy for RB. TPT induces potent cytotoxic effects in human RB cell lines at low nanomolar concentrations.\textsuperscript{17–21} In rodent models of RB, TPT significantly reduces tumor burden when delivered systemically\textsuperscript{15,20} or by repeated subconjunctival injections in aqueous media.\textsuperscript{20} Although data on TPT in human RB are scant, clinical responses to TPT have been reported in several patients with extraocular and recurrent intraocular RB.\textsuperscript{21–23} In this study, we evaluated the therapeutic effects of a single injection of subconjunctival TPT in FS in \textit{LH\textbeta}-\textit{Tag} mice.

**Materials and Methods**

**Growth Inhibitory Assays of TPT**

Y79 and \textit{Wer-Rb1} human RB cells were cultured in RPMI with 10\% fetal bovine serum and antibiotics. Cells were seeded in 96-well plates and treated with 1 to 1000 nM TPT (Hycamtin; GlaxoSmithKline, Philadelphia, PA). After 24, 48, 72, or 96 hours, cell viability was quantified by a cell proliferation assay (WST-1; Roche, Basel, Switzerland).

**Apoptosis Analysis of TPT**

Y79 cells were treated with 1 to 100 nM TPT. After 24 or 48 hours, caspase 3/7 activity was assayed by reagent (Caspase Glo 3/7; Promega, Madison, WI).

**Cell Cycle Distribution Analysis of TPT**

Y79 cells were treated with 30 nM TPT. After 24 hours, 5 \textmu{g}/mL propidium iodide (Sigma-Aldrich, Milwaukee, WI) and 10 \textmu{g}/mL fluorescent stain (Hoescht 33342; Calbiochem, San Diego, CA) were added, and cell cycle distribution was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Heidelberg, Germany).

**Growth Inhibitory Assays of TPT in FS**

Y79 cells were seeded into six-well plates and treated with TPT in FS, TPT in aqueous vehicle, FS only, or media only. Treatments were placed in transwell inserts with a 0.4-\textmu{m} pore plastic membrane (Falcon, BD Biosciences, Heidelberg, Germany) to simulate transscleral diffusion. After 48 or 72 hours, 100 \textmu{L} samples of cells in media were transferred to a 96-well plate, and growth inhibitory effects were quantified with a cell proliferation assay (WST-1; Roche).

**Fibrin Sealant**

FS (Tisseel VH; Baxter Healthcare, Deerfield, IL) consists of fibrinogen and thrombin in separate vials, plus separate solutions of fibrinolysis inhibitor and CaCl\textsubscript{2}. All components were heated to 37°C for 15 minutes in a heating/mixing appliance (Fibrinotherm; Baxter Healthcare, Deerfield, IL). The fibrinolysis inhibitor solution was then added to the fibrinogen and mixed in the device for 10 minutes. For the in vitro studies, the thrombin was reconstituted with CaCl\textsubscript{2} solution, and 100 \textmu{M} TPT (Hycamtin; GlaxoSmithKline) was added to obtain the desired drug concentrations. In the in vitro studies, the thrombin-CaCl\textsubscript{2} solution was injected into the vial of TPT and serially diluted to achieve the desired drug concentrations. FS was delivered with a device (Duopject; Tisseel VH; Baxter Healthcare, Deerfield, IL) that allows the plungers of two 1-mL syringes containing the separated FS components to be depressed simultaneously. The device includes a Y-shaped con-

Local Topotecan in Fibrin Sealant in Murine RB

Results

**Effects of TPT in Human RB Cells**

TPT induced potent, dose-dependent, growth-inhibitory effects in RB cells. The IC\textsubscript{50} in Y79 cells at 48 hours after treatment was approximately 35 nM. Cells treated with 100 nM TPT after 96 hours displayed nearly zero viability, as did cells treated with 1000 nM after 48, 72, and 96 hours (Fig. 1A). The IC\textsubscript{50} in \textit{Wer-RB1} cells after 48 hours of incubation was approx-

Animals

All animal procedures were approved by the UCSF Committee on Animal Research and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The \textit{LH\textbeta}-\textit{Tag} mouse model used in this study is heterozygous for a transgene composed of the simian virus 40 large T antigen gene (\textit{Tag}) driven by the human luteinizing hormone \textbeta-subunit promoter gene (\textit{LH\textbeta}).\textsuperscript{24} \textit{Tag} expression in the retina results in multifocal, bilateral retinal tumors analogous to human retinoblastomas.

Dose-Escalation Study

Eight groups of three 10- to 14-week-old wild-type mice were anesthetized and treated in both eyes with a single 30-\textmu{L} subconjunctival injection of TPT in FS (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg/mL). Mice were monitored for 5 weeks and killed. Eyes were enucleated, formalin fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Sections were examined by light microscopy. On completion of this study, two additional mice were treated bilaterally with 3.2 mg/mL TPT in FS to confirm the absence of intraocular toxicity at this dose.

Therapeutic Trial

Two randomized groups of 10-week-old \textit{LH\textbeta}-\textit{Tag} transgenic mice (20 in each group) were anesthetized and treated in the superior temporal subconjunctival space of the right eye with a single 30-\textmu{L} injection of 3.2 mg/mL TPT in FS or FS only using a 30-gauge needle. The contralateral eye in each group was left untreated to serve as an internal control. Thus, there were three control groups: eyes treated with FS, untreated eyes in TPT-treated mice, and untreated eyes in FS-treated mice. After 3 weeks, animals were killed, and eyes were serially sectioned and stained with hematoxylin and eosin. Approximately 100 sections were obtained from five levels throughout each eye.

Ocular Tumor Burden Analysis

Sections were analyzed in a masked fashion. For each eye, all sections from each of the five levels were examined by light microscopy. All tumor foci were imaged at 100× magnification, and the area of each focus was quantified in pixels using imaging software (Scion Image; Scion Corporation, Frederick, MD). Mean tumor burden per level per eye was calculated by summing the pixel count of all tumor foci and dividing by 5.

Statistical Analysis

An unpaired, two-tailed \textit{t} test was used to compare mean body weight change in treatment and control groups. To test for bilateral treatment effects, we performed an unpaired, two-tailed \textit{t} test to compare mean tumor burden per level in both eyes in TPT-treated and control mice. To test for local effects, we performed univariate analysis of variance (ANOVA) for differences in mean tumor burden per level among the four groups of eyes (eyes treated with TPT in FS, untreated eyes from TPT-treated mice, eyes treated with FS only, and untreated eyes from mice treated with FS only).
approximately 50 nM. Cells treated with 1000 nM demonstrated less than 20% viability after 48 hours and less than 10% viability after 72 and 96 hours (Fig. 1B). TPT also induced apoptotic effects in RB cells. After 48 hours, Y79 cells treated with 30 nM and 100 nM TPT demonstrated a 2.5-fold and a 6-fold increase in caspase-3/7 activity, respectively (Fig. 1C). Cell cycle analysis of TPT-treated Y79 cells showed an accumulation of cells in S and G2 (Fig. 1D). In cells treated with 30 nM TPT, cell cycle distributions for G0/G1, S, and G2/M phases were 9.7%, 35.7%, and 54.6%, respectively, whereas in vehicle-treated cells, distributions were 66.0%, 12.1%, and 21.8%, respectively. Y79 cells treated with TPT in FS also showed a dose-dependent reduction in cell viability after 48 and 72 hours (Fig. 2). Cytotoxic effects of TPT in FS did not differ significantly from the effects of TPT in aqueous vehicle (Fig. 2). The difference in cell viability between cells treated with FS only and untreated cells was negligible (data not shown).

**Dose-Escalation Study**

In the dose-escalation studies, clinical evidence of toxicity (mild eyelid alopecia in 2 of 10 eyes and mild enophthalmos in 1 of 10 eyes) was observed only in mice receiving the highest dose of 3.2 mg/mL. Mean weight change did not differ significantly between treatment and control groups. No histopathologic evidence of ocular toxicity was observed in any dose group. These data suggest that doses higher than 3.2 mg/mL would have been well tolerated. This dose was nevertheless chosen for the therapeutic study because TPT in FS proved to be viscous and technically infeasible to inject beyond this dose.
Therapeutic Study

Toxicity. In the therapeutic trial, 3 of 20 eyes treated with TPT in FS developed mild enophthalmos. Among eyes treated with FS only, one eye developed hemorrhage that later progressed to phthisis bulbii, and 4 of 20 eyes demonstrated media opacification. Two of these four eyes became phthbirs. These injuries were mostly likely the result of inadvertent globe puncture during subconjunctival injection. Mean weight change did not differ significantly between treatment and control groups. No histopathologic evidence of toxicity was noted in treated or untreated eyes from TPT-treated mice. Histopathologic analysis of eyes treated with FS revealed phthisis bulbii in three eyes and cataractous changes in two additional eyes that had demonstrated media opacification during treatment. No histopathologic evidence of toxicity was noted in untreated eyes from the same mice.

Tumor Burden Analysis. Six eyes from three mice treated with FS only were excluded from analysis because of phthisis changes in the treated eye and early killing, as required by our animal use protocol. Additionally, one eye treated with FS only was excluded from analysis because retinal tissue was lost in processing. Therefore, 33 of 40 eyes in FS-only treated mice were included in the final analysis: 16 eyes treated with FS only and 17 untreated eyes.

Three eyes in TPT-treated mice were also excluded from analysis. One untreated eye was excluded because retinal tissue was lost in processing. In addition, both eyes from one TPT-treated mouse were excluded because of abnormally high tumor burden. Mean tumor burden in each of these two eyes was greater than 800,000 pixels, or >4 SD from the mean in their respective treatment groups (eyes treated with TPT in FS and untreated eyes from the same mice). Both eyes were therefore excluded from statistical analysis as outliers.25 As a result, 37 of 40 eyes in TPT treated mice were included in the final analysis: 19 eyes treated with TPT in FS and 18 untreated eyes.

The results of ocular tumor burden analyses are summarized in Table 1 and Figure 3. Mean tumor burden in TPT-treated and sealant-treated control eyes is depicted in representative sections in Figure 4. Comparison of mean tumor burden in both eyes from TPT-treated and from control mice demonstrated a statistically significant reduction in tumor burden (44,698 ± 40,467 pixels vs. 109,586 ± 158,887 pixels; P = 0.04; Fig. 3). Mean tumor burden did not differ significantly in eyes treated with TPT in FS and untreated contralateral eyes (P = 0.54). A trend toward statistically significant reduction in tumor burden was observed in eyes treated with TPT in FS compared with eyes treated with FS only and untreated eyes from control mice (P = 0.11 for both comparisons). A similar trend in tumor burden reduction was observed in untreated eyes from TPT treated mice compared with eyes treated with FS only (P = 0.25) and untreated eyes from control mice (P = 0.19).

Discussion

In this study, we investigated the therapeutic effects of a single subconjunctival injection of TPT in FS in LHB-Tag transgenic murine RB. Treatment resulted in a bilateral reduction of tumor burden without a significant difference between treated and untreated eyes. These results suggest that drug was delivered to both eyes predominantly through the hematogenous route. This conclusion is consistent with a recent study in rabbits by Carcaboso et al.,26 who report exclusively systemic delivery of TPT after pericuclar administration in aqueous vehicle. These researchers observed comparable vitreous area under the curve (AUC) values for lactone (active) TPT in treated eyes, untreated eyes, and eyes of control animals injected intravitreally with the same dose. Interestingly, pericuclar administration resulted in significantly lower plasma AUC values, suggesting that this approach could provide therapeutic benefits comparable to those of intravenous administration while also reducing systemic toxicities. The risk for local toxicities would have to be thoroughly evaluated before pericuclar TPT could be considered a feasible alternative to intravenous therapy. This risk could be minimal; Carcaboso et al.26 observed no local toxicities in rabbits treated by this approach. Additional clinical benefits could be obtained by pericuclar administration in a controlled-release vehicle such as FS.

The efficiency of transscleral drug delivery is determined by the relative rates of transscleral diffusion and drug clearance by local vasculature. The transscleral diffusion rate of a drug depends strongly on its molecular weight,27,28 molecular radius,29 and solubility.28 Molecular weight and radius are inversely correlated with scleral permeability because an agent must diffuse through the porous collagen fiber matrix of the sclera.30 Hydrophilic compounds cross the sclera more easily than lipophilic compounds.31 Passage occurs by passive diffu-
sion through the aqueous media of the sclera, a largely acellular tissue containing approximately 70% water.\(^{30}\) TPT should have a favorable transscleral diffusion rate because its molecular mass, molecular radius, and hydrophilicity are in the range of other small molecules that readily cross the sclera in vitro, including carboplatin,\(^4\) fluorescein,\(^7\) and rhodamine 6G.\(^{31}\) (Molecular weight, molecular radius, and solubility are as follows: TPT, 421 g/mol, 6.9 Å, 1 mg/mL; carboplatin, 371 g/mol, 3.9 Å, 14 mg/mL; fluorescein, 332 g/mol, 4.8 Å, 0.6 g/mL; rhodamine 6G, 479 g/mol, 6.9 Å, 10 mg/mL. Values were obtained from the literature, except for molecular radii of TPT, carboplatin, and rhodamine 6G, which were calculated using ChemDraw 3D [CambridgeSoft Corporation, Cambridge, MA]).

Carboplatin\(^4\) and fluorescein\(^7\) also diffuse through the sclera at physiological levels when delivered locally in vivo. Compared with intravenous delivery, periocular injection of these agents results in significantly greater peak drug levels at later time points in the vitreous and choroid/retina, indicating that the major route of drug delivery to these tissues after local injection is transscleral rather than hematogenous. We have confirmed these results in our study of subconjunctival carboplatin in FS in transgenic murine RB, in which we observed a profound local treatment effect (95% reduction in tumor burden in treated eyes compared with untreated eyes in the same mouse; \(P < 0.004\)).\(^{16}\) The results of the present study and the pharmacokinetic study by Carcaboso et al.\(^{20}\) suggest that, despite having similar diffusion properties, TPT does not cross the sclera as efficiently as carboplatin in vivo because it is cleared by local vasculature at a significantly higher rate than carboplatin.

Li et al.\(^7\) first demonstrated that clearance by local vasculature can present a barrier to transscleral drug delivery. They found that agents that fail to penetrate the sclera when injected periocularly in vivo diffuse efficiently when injected postmortem, after vascular clearance has been terminated. Carcaboso et al.\(^{20}\) obtained similar results in rabbits injected with periocular TPT, confirming that transscleral diffusion of this agent is prevented in vivo by rapid clearance from the periocular space. Other groups have shown that the conjunctival blood and lymphatic vasculature play a more important role in drug clearance than the choroid\(^7\) and that local clearance rates vary by the site of periocular injection.\(^7\) The rate of drug clearance is higher in the retrobulbar space than in the Tenon capsule because of greater exposure to orbital tissues.\(^7\)

Properties that determine an agent’s vascular clearance rate after periocular injection have not been clearly elucidated. Kompella et al.\(^8\) have proposed that slow vascular clearance of subconjunctival budesonide may be attributed to extensive binding of this drug to local tissues. This hypothesis is consistent with the extensive serum protein binding of budesonide (85%–90%\(^33\)). However, serum protein binding does not predict the relative clearance rates of carboplatin and TPT. Although carboplatin is cleared more slowly by periocular vasculature than TPT, plasma protein binding of TPT (35%\(^34\)) is actually higher than that of carboplatin (negligible after 4-hour exposure\(^35\)). On the other hand, it is possible that serum protein binding by an agent does not accurately reflect periocular tissue binding because drugs could bind extensively to local proteins, such as scleral matrix proteins, that are scarce or absent in serum.

For any sustained release system, drug must be released into the periocular space at a rate in excess of the blood and lymphatic vascular clearance rate for any transscleral drug diffusion to occur. In some systems, local retinal and vitreal drug delivery can be achieved only by exceeding a threshold dose. For example, most patients receiving a periocular injection of ≤5 mg triamcinolone acetonide suspension have undetectable vitreal drug levels at a median of 5.5 days after treatment.\(^36\) In contrast, patients receiving a 40-mg dose have detectable vitreal drug levels up to 4 weeks later.\(^37\) Similar results have been shown in rabbits receiving 10- and 20-mg periocular injections of triamcinolone acetonide. After 3 hours, only the latter dose resulted in detectable drug levels, indicating that the drug release rate at the latter dose exceeded the vascular clearance rate.\(^6\)
Our data suggest that, at the dose we chose, the release rate of TPT from FS did not exceed the vascular clearance rate. It is conceivable that significant local effects could have been achieved in this study by overloading the FS with more TPT. However, this would have required the use of a different formulation of TPT, which proved to be viscous and difficult to inject at the dose selected for the study (3.2 mg/mL). Higher dosing was infeasible, possibly on account of the high fraction of inactive ingredients (mannitol and tartaric acid) in pharmaceutical TPT. Although the chosen dose induced only minimal toxicities, we reasoned that it would suffice to demonstrate treatment effects because it was already extremely high relative to the systemic dose given to children with RB (2 mg/m² per day for 5 consecutive days per cycle). Assuming that the average weight of a mouse is 0.025 kg and that the average height and weight of a 1-year-old child are 75 cm and 10 kg, with an average body surface area of 0.5 m², mice in this study received a subconjunctival dose of TPT 40-fold greater per kilogram of body weight than the daily intravenous dose an infant with RB would receive. This amounts to a subconjunctival dose of TPT in a mouse equivalent to one tenth the total dose an infant with RB would receive intravenously (0.1 mg vs. 1 mg). The absence of intraocular toxicities in eyes treated with such a high dose of TPT in FS prompted us to examine whether FS could be diminishing the activity of TPT. The results of our in vitro studies, demonstrating comparable cytotoxic effects with TPT in aqueous media and equidose TPT in FS, ruled out that possibility. The IC₅₀ of TPT in RB cells was in the low nanomolar range, and growth-inhibitory effects were associated with apoptosis induction and accumulation of cells in S and G₂, consistent with previous reports.

Preferential absorbance of subconjunctival TPT into the general circulation has significant clinical implications. Laurie et al. have reported in a xenograft model of RB that systemic carboplatin and TPT therapy was the most effective among several chemotherapeutic combinations tested, including carboplatin, etoposide, vincristine (CEV). In light of these results, they suggest that TPT may be a suitable replacement for etoposide in CEV therapy for RB. However, as they discuss, combined treatment with the topoisomerase I-inhibitor topotecan and TPT therapy was the most effective among several chemotherapeutic combinations tested, including carboplatin and TPT therapy. It may be preferable to administer combination carboplatin and TPT therapy by delivering carboplatin or both agents locally. In developing an appropriate protocol, it would be important to determine the drug plasma levels associated with local administration of each agent.

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