Topotecan Vitreous Levels after Periocular or Intravenous Delivery in Rabbits: An Alternative for Retinoblastoma Chemotherapy

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PURPOSE. To determine the extent and the mechanism by which topotecan, a candidate agent for the treatment of retinoblastoma, gains access to the vitreous when administered by periocular injection or intravenous infusion.

METHODS. In vivo experiments were conducted in which albino rabbits received 1 mg topotecan by periocular injection (POI group; n = 30) or as a 30-minute intravenous infusion (IV group; n = 16). Plasma and vitreal topotecan concentrations were analyzed during the 10 hours after administration. A population pharmacokinetic model was fit to the data. Additionally, periocular injections were performed postmortem to study the effect of removing the blood vasculature barrier.

RESULTS. Potentially active lactone topotecan levels were detected in the vitreous in the POI and IV groups. Both administration schedules induced high total topotecan plasma exposures because of absorption from the periocular depot, though plasma lactone area under the curve (AUC) was significantly higher in the IV group. Similar vitreal concentrations were found in treated and control eyes in the POI group. The transfer from the periocular compartment to the vitreous was negligible. The absence of drug levels in the control eye of the postmortem-injected rabbits confirmed the systemic delivery of topotecan. Local toxicity was not observed.

CONCLUSIONS. As a consequence of a favored passage across the blood–retinal barrier, considerable topotecan vitreous levels were detected in a rabbit model after systemic or periocular administration. Transscleral entry in vivo was constrained by rapid clearance from the administration site. (Invest Ophthalmol Vis Sci. 2007;48:3761–3767) DOI:10.1167/iovs.06-1152

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Chemotherapy is now routinely given to reduce the tumor volume of intraocular retinoblastoma in the hope of avoiding external beam irradiation because of the documented association between external beam irradiation in children with retinoblastoma in the first year of life and the subsequent development of second, nonocular cancers. Carboplatin-based regimens are frequently used, often in combination with vincristine and etoposide, for tumor chemoreduction. However, this treatment has been less successful in treating eyes with vitreous seeding, even when external beam radiation is added. Poor penetration of chemotherapy drugs to the vascular vitreous may be the major reason for treatment failure. To overcome this, clinicians may choose to intensify systemic chemotherapy, but this would expose these susceptible patients, who are likely to become long-term survivors, to greater short- and long-term toxicity, including chemotherapy-associated leukemia. Periocular administration of chemotherapy may be an option to deliver higher concentrations of chemotherapy drugs to the posterior segments of the eye. This strategy would potentially improve ocular drug penetration by the transscleral pathway, avoiding the inner blood–retinal barrier restriction (passage through the retinal vessel walls) to the systemic drug delivery. Carboplatin is one of the few chemotherapy agents for which the local route has been thoroughly studied as an alternative to the systemic administration. However, periocular carboplatin results in frequent acute and uncomfortable toxicity, and severe long-term sequelae have even been observed. The identification of newer drugs with different mechanisms of action, innovative delivery systems, and better toxicity profiles, suitable for the treatment of retinoblastoma, is an area of intense research. Innovative delivery systems such as episcleral exoplants and fibrin sealants for such commonly used drugs as carboplatin, targeting the tumor vasculature with agents such as combretastatin A-4 prodrug, and proton radiation therapy have all recently been investigated in this context.

Topotecan has shown promising activity in selected patients with retinoblastoma, and recent studies have shown its high activity in vitro and in vivo in rodent retinoblastoma models in combination with carboplatin and inducers of the p53 pathway. The association of carboplati...
ocular pharmacokinetics of intravenous topotecan in rats, showing good penetration to the vitreous after intravenous injection of maximum tolerated doses of 2 mg/kg body weight. That study provided the background for the current clinical trial at St. Jude Children’s Research Hospital for patients with vitreous seeding, including systemic topotecan and periocular carboplatin for those at higher risk. Because the systemic toxicity of carboplatin in effective doses for retinoblastoma is lower than that of topotecan, an alternative way would be to combine periocular topotecan and systemic carboplatin. However, no information is available about the use of periocular topotecan. To further study the ocular penetration of topotecan in a preclinical model, we tested its in vivo ocular and plasma pharmacokinetic profiles by the periocular route and compared the results with conventional intravenous infusion. Additionally, to explain the mechanisms involved in topotecan ocular distribution, we performed postmortem experiments to inhibit conjunctival vasculature clearance and hematogenous delivery of the drug.

**Materials and Methods**

Commercial formulations of topotecan (Hycamtin, 4 mg per vial) and topotecan hydrochloride standard were kindly supplied by GlaxoSmithKline (Buenos Aires, Argentina). Commercial topotecan is formulated as a mixture of drug with tartaric acid that confers an acidic pH to the reconstituted solution. At physiological pH, the active form of topotecan, a closed lactone ring, is reversibly hydrolyzed to a carboxylate inactive form.24

**In Vivo Studies**

New Zealand albino rabbits weighing 1.8 to 2.2 kg each were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were anesthetized intramuscularly with a mixture of ketamine (37.5 mg/kg) and xylazine (5 mg/kg) throughout the experiment. Topical proparacaine eye drops (0.5%) were used to anesthetize the ocular surface before periocular injection or eye puncture. Animals were humanely killed with a rapid intravenous bolus injection of sodium thiopental (100 mg).

For the in vivo studies, 46 rabbits were included and divided into two groups. The first group (periocular injection [POI group], n = 30) received periocular injections of topotecan (1 mg in 1 mL saline solution) with a 25-gauge needle in the inferior temporal quadrant of the right orbit (left orbits were not injected). The absence of leakage from the injection site was verified. The second group (intravenous infusion [IV group], n = 16) received intravenous 30-minute topotecan infusion (1 mg in 5 mL saline solution) through the infusion pump after a central jugular catheter was placed in each.

**Sampling Schedule**

For the ocular studies, 150 to 200 µL vitreous humor was aspirated from the inner region of the vitreous chamber with a 18-gauge needle inserted in the superior region of the sclera approximately 3 mm away from the limbus and 180” away from the injection site. Translucent vitreous samples were taken at 15, 30, 60, 120, 240, 360, and 600 minutes after injection (POI group) and at 0, 15, 30, 45, 60, 120, 240, 360, and 600 minutes after the completion of the infusion (IV group).

Each eye was punctured only once. More samplings might have led to overestimation of the topotecan entrance to the eye because of the previous rupture of the ocular blood barrier and the changes in ocular dynamics. This method for procurement of the vitreous enabled us to better characterize in vivo the real-time pharmacokinetic profile of topotecan lactone and to avoid time-dependent hydrolysis to the carboxylate form that might have occurred if the vitreous had been procured by other time-consuming methods, such as eye removal and subsequent dissection after freezing. After homogenizing the samples with a vortex, 50 µL vitreous was spiked with 200 µL cold methanol and was again vortexed to precipitate the proteins, centrifuged at 8000 rpm for 5 minutes, and injected into the chromatographic system.

For the plasmatic studies, samples were taken from the ear veins of noncannulated rabbits (POI group) at 5, 15, 30, 45, 60, 90, 120, 180, 240, and 300 minutes. Samples of cannulated jugular veins (IV group) were taken from rabbits at 10, 20, 30, 45, 60, 90, 120, 150, 270, and 390 minutes after initiation of the topotecan infusion. The first two samples were collected from the ear veins, and the rest were taken from the catheter after the IV tube was carefully rinsed. At each time point, 1 mL blood was collected, heparinized, and centrifuged at 3000 rpm for 5 minutes. Plasma samples (100 µL) were mixed with 400 µL cold methanol, vortexed, and centrifuged at 8000 rpm for 5 minutes, and supernatants were injected in the chromatographic system.

Enucleated eyes underwent routine histopathology examination by an experienced pathologist. Vitreous samples were not taken from two rabbits of the POI group; instead, they were observed for 1 week and underwent bilateral enucleation after funduscopiy.

**Postmortem Studies**

Albino rabbits (n = 4) obtained from innocuous nonocular research studies at the University of Buenos Aires were anesthetized and humanely killed. Fifteen minutes after death, 1 mg topotecan was injected into the right orbit, as previously described. Animals were maintained in the prone position under a heating lamp until sampling time. All eyes were punctured 2 hours after administration and were immediately assayed for vitreous topotecan concentrations.

**HPLC Method**

Topotecan concentrations were determined by high-performance liquid chromatography (HPLC) analysis according to a method modified...
from Warner and Burke. Briefly, the chromatographic system consisted of an HPLC pump (Waters 515; Waters, Milford, MA) and a fluorometric detector (FL-45A; Bioanalytical Systems, West Lafayette, IN) with an excitation wavelength of 368 nm and an emission wavelength of 592 nm. We used a reverse-phase 3-μm, 3 × 150-mm column (C18; Phenomenex Co., Torrance, CA). Samples (20 μL) were injected at a flow rate of 0.4 mL/min at room temperature. Retention times of carboxylate and lactone topotecan were 3.5 and 8.4 minutes, respectively.

For the preparation of topotecan standards, stock solutions of 1 mg/mL topotecan hydrochloride were prepared in methanol and stored at −20°C. Topotecan lactone and carboxylate working solutions of 500 μg/mL were obtained by mixing equal volumes of the topotecan stock solution with pH 3 or pH 10 phosphate buffer, respectively. These solutions were maintained for 30 minutes at room temperature before further processing to ensure conversion to the pH-dependent forms of the drug.

**Pharmacokinetic Analysis**

The population pharmacokinetic model was fit to total topotecan concentrations measured for all animals (Nonlinear Mixed Effects Modeling; NONMEM Project Group, University of California, San Francisco, CA). Plasma concentrations after intravenous infusion were evaluated for the best fit and consequently were modeled according to a two-compartment open model. Elimination rate constant (k10), transfer rate constants between central and peripheral compartments (k12 and k21), and volume of the central compartment (Vc) were estimated by this approach. These parameters were fixed for further evaluation of the parameters describing vitreous total topotecan concentrations after intravenous and periocular administrations. As displayed in Figure 1, a vitreous compartment was then added to both models, and a fourth hypothetical compartment (periocular) was included to simulate the orbital space in the periocular administration. The volume of the vitreous compartment was fixed at 1.4 mL according to published data.

Area under the curve (AUC) of each topotecan level in plasma and vitreous were calculated by the linear trapezoidal rule with the formula

$$AUC = \sum_{i=2}^{n} \frac{(c_i + c_{i-1}) \Delta t_i}{2}$$

where $c_i$ is the mean concentration at the $i^{th}$ time point (measured in ng/mL) and $\Delta t_i$ are time intervals, with $\Delta t_n = 0$.

The SE of the AUC (SEAUC) was calculated with the law of propagation of errors as

$$SEAUC = \sqrt{\sum_{i=1}^{n} (\frac{(c_i + c_{i-1})^2 \Delta t_i^2}{4})}$$

in which $SE_i$ is the variance of the mean concentration at the $i^{th}$ time point.

**Statistical Analysis**

Average values are represented as mean ± SD. Normal distribution of samples and homogeneity of variance were confirmed by the Shapiro-Wilk trial and the Levene test, respectively. The Student’s t-test was used to determine significance between mean values. $P < 0.05$ was considered significant.

**RESULTS**

**In Vivo Studies**

Figure 2 shows vitreous levels of lactone and total topotecan in the albino rabbits. After periocular injections of topotecan in the right orbits, vitreous lactone levels in the range of 5 to 10 ng/mL were achieved in the right vitreous and maintained until 4 hours after administration (Fig. 2A). The contralateral eye presented a similar concentration profile (Fig. 2B). Intravenous infusion of topotecan induced peak lactone levels between 5 and 10 ng/mL that decreased to lower than 5 ng/mL by 2 hours after infusion (Fig. 2C).

Topotecan plasma profiles are displayed in Figure 3. As could be expected, the periocular total topotecan data curve

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933443/ on 10/02/2017)
resembled a typical absorptive phase followed by an elimination phase, whereas the intravenous data showed maximum concentrations at the end of the 30-minute infusion that decreased within the first hour after infusion (Fig. 3A). Lactone plasma profiles are depicted in Figure 3B.

**Pharmacokinetics**

Pharmacokinetic parameters describing total topotecan disposition were estimated (Table 1). Modeling of topotecan concentration profiles resulted in a good correlation between observed and predicted concentrations over time (Fig. 2). Vitreous lactone topotecan AUC values were similar for treated and control eyes in the POI group, and these values were comparable to those obtained after intravenous infusion (Table 2). Intravenous infusions elicited higher total topotecan vitreous AUC values than did periocular injections (P < 0.01; t-test). Interestingly, though total topotecan plasma AUC levels were similar for both groups, lactone plasma AUC levels were higher for the IV group (P < 0.01; t-test).

**Postmortem Studies**

Two hours after periocular administration, mean postmortem lactone levels in the right eyes were 44 ng/mL (eight times higher than in vivo concentrations at the same point). The postmortem ratio of lactone to total topotecan was 0.8 for rabbits, twice the in vivo ratio at the same time point (0.4). Topotecan levels were below the detection limit in all contralateral eyes.

**Toxicity**

Mild periorbital edema occurred in most animals after periocular administration that resolved spontaneously. No evidence of local or funduscopic toxicity was found 7 days after topotecan injection. No histopathologic evidence of toxicity was observed in any animal.

**DISCUSSION**

Recently, topotecan was reported to have potent and fast activity against human retinoblastoma in vitro.20 Low concentrations (8–13 ng/mL) of topotecan were required to reduce Weri1 or Y79 retinoblastoma cell line viability by 50% within only 15 minutes of exposure. This fast action is equivalent to that observed with carboplatin but contrasts with the higher times of exposure needed to reach the same effect with vincristine and etoposide (4 and 6 hours, respectively). These promising experiments also showed that the combination of topotecan and carboplatin was a highly effective schema in animal models,20 supporting the proposal of combination therapy with topotecan and carboplatin for the treatment of retinoblastoma as an alternative to the widely used triple-drug therapy (carboplatin + etoposide + vincristine). However, the systemic use of both agents is limited by severe hematopoietic toxicity.

Although chemotherapy prompt researchers to seek alternative means of achieving desirable concentrations in the tumor tissue, minimizing systemic exposure to the drug. One strategy to improve the delivery of chemotherapy agents to the vitreous is to administer the drug by way of the periocular route. In the present study, we explored the ability of topotecan to gain access to the vitreous in rabbits, a proper animal model for ocular pharmacokinetic studies. The potential of the periocular and intravenous routes of administration of topotecan to do so was assessed.

Lactone topotecan levels at potentially active concentrations were detected in the vitreous in our experiments. Total topotecan levels achieved in our study were within the range of concentrations reported to obtain fast reduction of cell viability in retinoblastoma cell lines,20 and potentially active concentrations were attainable up to 6 hours after injection. Although considerable topotecan vitreous levels were detected in the treated eye, the following observations from our study confirmed topotecan vitreal penetration in rabbits after periocular injection was primarily hematogenous (through the blood-ocular barrier) rather than transscleral: (1) comparable vitreous concentration profiles and lactone AUC values achieved by periocular and intravenous schedules (Figs. 2A, 2C; Table 2); (2) similar concentrations in treated and control eyes in the periocular group (Fig. 2A, 2B); (3) complete sys-

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**Table 1. Pharmacokinetic Parameter Estimates Generated from NONMEM Modeling**

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
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<td>Vc (L)</td>
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**Figure 3.** Plasma levels of total topotecan (A) and lactone (B) after periocular injection (circles) and 30-minute intravenous infusion (triangles) in rabbits. Displayed data are mean ± SD of 5 to 10 individual values. For periocular studies, each rabbit ($n = 20$) was sampled at three time points from the ear vein. For intravenous studies, each animal ($n = 10$) was sampled during the whole study using an intravenous catheter.
temic absorption after periorcular injection, leading to plasma total topotecan AUCs equivalent to those obtained with the intravenous topotecan (Table 2); (4) negligible $k_{es}$ (transfer rate constant from the periocular compartment to the vitreous) compared with the $k_{dl}$ (from the periocular to the central compartment; Table 1); and (5) absence of drug levels in the control eyes of the rabbits injected postmortem.

Differences in total topotecan vitreous AUC values between IV and POI groups could be attributed to a greater concentration gradient between plasma and the posterior segment in the IV group, which would have enhanced ocular absorption of the drug. In parallel, similar vitreous lactone AUCs in both groups might have been attributed to sustained lactone delivery from the injection site in the POI group as opposed to rapid first-order conversion kinetics to carboxylate in the IV group.29

In fact, though lactone plasma AUC from $t_0$ to the last time point was lower for the POI group than for the IV group (Table 2), lactone AUC from 1.5 hours to the last sampling time was significantly higher ($4.0 \pm 4.3$ and $27.5 \pm 3.1$ ng·h/mL for POI and IV groups, respectively; $P < 0.01$; $t$-test). The non-charged nature of the lactone form compared with the charged carboxylate form favored the passage of the lactone across the membranes. In this way, the lactone moiety could be absorbed in the POI group for a longer period, and the AUC value might reach a value comparable to that calculated for the IV group.

The mechanisms by which such topotecan in vivo pharmacokinetic results were obtained in our experiments lay in the complete understanding of the ocular barriers and the penetration routes after transscleral delivery of the drugs, which have been only partially elucidated.7,9,39 Postmortem experiments, by interrupting the normal circulation of the animals, helped to verify the effect of the conjunctival and choroidal barriers and to enhance drug permeation on the injected eye to the levels expected to be obtained by applying in vitro permeability coefficients.30,31 Similarly, ex vivo experiments with enucleated eyes have been performed with similar purpose and show significant increases in vitreous drug concentrations compared with in vivo assays.32 Topotecan clearance would also be favored by the dispersion of the injected volume throughout the periorcular space, enhancing surface contact with the absorptive conjunctival tissue. We postulate that injection volumes in the range used in our study (1 mL) would likely be distributed in the whole available space in the orbit; thus, we use the term periorcular to include all the possible intraorbital locations, including peribulbar, subconjunctival, and intracapsular. Consequently, we assume that the observed interindividual variations among the pharmacokinetic data of our study could be ascribed to different distributions of the injected volume into the orbit.

Anatomo-physiological differences between rabbits and humans, such as the greater peripheral choroidal flow of the rabbit eye compared with the primate eye flow,33 could hinder transscleral drug penetration and induce a bias toward systemic absorption in the rabbit model, leading to different distributions of the same drug in human and rabbit periorcular studies.34–36 Regarding the rabbit retinal vasculature, with blood vessels only in the basal surface, differences between deeply vascularized human and rat retinas could be the reason for different vitreous-to-plasma topotecan AUC ratios found in our study (AUC vitreous/plasma = 0.29) compared with previous studies in rats by the IV route (AUC vitreous/plasma = 0.38).20 In addition, as mentioned, altered ocular barriers are expected in human retinoblastoma–bearing eyes. Only clinical trials or an adequate animal retinoblastoma model will provide enough data to extrapolate our results to the clinic.

If our findings in this non–tumor-bearing animal model also occur in children with retinoblastoma, a significant amount of periorcularly administered topotecan may reach the systemic circulation, raising concerns about possible systemic toxicity. Myelosuppression is the dose-limiting toxicity of topotecan given intravenously and correlates with systemic exposure to the lactone moiety of the drug.27 However, the cumulative systemic exposure obtained with a 5-day short infusion schedule or a 21-day continuous infusion is higher than that achieved with a single dose of periorcular topotecan. Therefore, as reported recently by Laurie et al.,21 systemic toxicity after periorcular topotecan is unlikely. Moreover, the lactone plasma AUC for our POI group was lower than for the IV group, indicating a lower systemic exposure to the topotecan form that causes most toxic effects. Nevertheless, plasmatic topotecan levels will be measured in children with retinoblastoma who receive periorcular topotecan in our future phase I trial.

Why some drugs cross the sclera efficiently after local administration while others are rapidly cleared is difficult to answer, though a potential explanation is provided by the individual drug properties. A poorly soluble drug such as celecoxib, when administered periorcularly in rats as a suspension, induced 100 times higher concentrations than those obtained after systemic injection, probably because periorcular clearance mechanisms were saturated by the local drug depot.38 Vitreous levels in the contralateral eye were low and comparable to those induced by the systemic route.38 Subconjunctival administration of a solution of budesonide, a lipophilic drug, induced detectable levels in the treated eye for up to 3 days.39 Binding to local tissue was proposed to enhance and sustain transscleral delivery, whereas systemic delivery was negligible because budesonide levels in the opposite eye were undetectable.39 Similarly, periorcular carboplatin solutions are likely to accumulate in the injection site, probably because of local protein binding or drug precipitation, reaching high transscleral delivery, in contrast to low systemic absorption.10,40 Carboplatin formulated as a suspension enclosed in fibrin sealant has been shown to induce high drug levels in the exposed sclera for up to 2 weeks,41 possibly by the interaction between carboplatin and fibrinogen, leading to the formation of nanoparticles that remain in the fibrin mesh after clot formation.42 Preliminary assays were performed by our group to determine the ability of fibrin sealant to entrap topotecan. The absence of

### Table 2. Plasma and Vitreous AUC Values (ng·h/mL) after Intravenous and Periorcular Administrations of Topotecan

<table>
<thead>
<tr>
<th></th>
<th>Plasma (Control Eye)</th>
<th>Vitreous (Control Eye)</th>
<th>Vitreous (Treated Eye)</th>
<th>AUC Ratio* (Vitreous/Plasma)</th>
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<tbody>
<tr>
<td></td>
<td>Total Lactone</td>
<td>Total Lactone</td>
<td>Total Lactone</td>
<td></td>
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<tr>
<td>Intravenous</td>
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<tr>
<td>Lactone</td>
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<td>$143.5 \pm 4.1$†</td>
<td>$113.6 \pm 6.7$†</td>
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<td>Vitreous</td>
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<td>$108.8 \pm 4.8$</td>
<td>$76.7 \pm 7.5$</td>
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<tr>
<td>Periocular</td>
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<tr>
<td>Lactone</td>
<td>$362.8 \pm 8.2$</td>
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</table>

Topotecan was administered at 1 mg/rabbit. Data are expressed as mean ± SE, as calculated by the linear trapezoidal rule.

* Vitreous-to-plasma lactone AUC ratio.
† Statistically significant differences ($P < 0.01$).
fibrin clot formation was observed when commercial or reference topotecan (1 and 5 mg/mL, respectively) was added to the fibrinogen or thrombin solutions.

For drugs showing rapid systemic clearance after periocular application,13-16,34,36,43,44 the quest for appropriate delivery systems seemed to be essential to prolong drug–sclera contact times. However, periocular prolonged-release devices may also lead to ineffective transscleral delivery because of preferential systemic absorption.13-32 Thus, sustained release would be translated to sustained periocular clearance unless specific strategies were applied to isolate the system from the conjunctival tissue. Specially designed coated implants have been postulated to solve this problem with moderate success.44-45

The need for transscleral selective implants is especially critical for systemic toxic drugs such as topotecan. Future developments by our group will be focused on this issue.

To conclude, periocular or intravenous administration of 1 mg topotecan reached potentially active vitreous levels of its lactone moiety in a rabbit model. Our results show that systemic absorption accounts for most topotecan vitreous delivery in vivo in this non–tumor-bearing model. Additional experiments to find the best way to find a predominant transscleral pathway are warranted.

References


38. Ayalasomayajula SP, Kompella UB. Retinal delivery of celecoxib is several-fold higher following subconjunctival administration compared to systemic administration. *Pharm Res*. 2004;21:1797–1804.


