Experimental Eye Research 108 (2013) 103–109

Contents lists available at SciVerse ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Ocular and systemic toxicity of intravitreal topotecan in rabbits for potential treatment of retinoblastoma

Emiliano Buitrago [a], María José Del Sole [b], Ana Torbidoni [c], Adriana Fandino [g], Marcelo Asprea [d], J.O. Croxatto [e], Guillermo L. Chantada [c], Guillermo F. Bramuglia [a,1], Paula Schaiquevich [f,*,1]

[a] Department of Pharmacology, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina
[b] Pharmacology Laboratory, CIVETAN-CONICET, Faculty of Veterinary, National University of the Centre of Buenos Aires, Tandil, Argentina
[c] Service of Hematology-Oncology, Hospital de Pediatria JP Garrahan, Buenos Aires, Argentina
[d] Laboratory of Animal Care, Department of Laboratory Services, Hospital de Pediatría JP Garrahan, Buenos Aires, Argentina
[e] Fundación Oftalmológica Argentina Jorge Malbrán, Buenos Aires, Argentina
[f] CONICET-Clinical Pharmacokinetics Unit, Hospital de Pediatría JP Garrahan, Buenos Aires, Combate de los pozos 1881, Ciudad de Buenos Aires, CP 1245 Buenos Aires, Argentina
[g] Service of Ophthalmology, Hospital de Pediatria JP Garrahan, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 23 August 2012
Accepted in revised form 2 January 2013
Available online 16 January 2013

Keywords:
intravitreal
topotecan
rabbits
retinoblastoma

ABSTRACT

Treatment of intraocular retinoblastoma with vitreous seeding is a challenge. Different routes of chemotherapy administration have been explored in order to attaining pharmacological concentrations into the posterior chamber. Intravitreal drug injection is a promissing route for maximum bioavailability to the vitreous but it requires a well defined dose for achieving tumor control while limited toxicity to the retina. Topotecan proved to be a promising agent for retinoblastoma treatment due to its pharmacological activity and limited toxicity. High and prolonged concentrations were achieved in the rabbit vitreous after 5 μg of intravitreal topotecan. However, whether a lower dose could achieve potentially therapeutic levels remained to be determined. Thus, we here study the pharmacokinetics of topotecan after 0.5 μg and the toxicity profile of intravitreal topotecan in the rabbit eye as a potential treatment of retinoblastoma. A cohort of rabbits was used to study topotecan disposition in the vitreous after a single dose of 0.5 μg of intravitreal topotecan. In addition, an independent cohort of non-tumor bearing rabbits was employed to evaluate the clinical and retinal toxicity after four weekly injections of two different doses of intravitreal topotecan (Group A, 5 μg/dose; Group B, 0.5 μg/dose) to the right eye of each animal. The same volume (0.1 ml) of normal saline was administered to the left eye as control. A third group of rabbits (Group C) served as double control (both eyes injected with normal saline). Animals were weekly evaluated for clinical and hematologic values and ocular evaluations were performed with an inverse ophthalmoscope to establish potential topotecan toxicity. Weekly controls included topotecan quantitation in plasma of all rabbits. Electroretinograms (ERGs) were recorded before and after topotecan doses. One week after the last injection, topotecan concentrations were measured in vitreous of all eyes and samples for retinal histology were obtained. Our results indicate that topotecan shows non linear pharmacokinetics after a single intravitreal dose in the range of 0.5–5 μg in the rabbit. Vitreous concentration of lactone topotecan was close to the concentration assumed to be therapeutically active after 5 h of 0.5 μg intravitreal administration. Eyes injected with four weekly doses of topotecan (0.5 or 5 μg/dose) showed no significant differences in their ERG wave amplitudes and implicit times in comparison with control ($p > 0.05$). Animals showed no weight, hair loss or significant changes in hematologic values during the study period. There were no significant histologic damage of the retinas exposed to topotecan treatments. After intravitreal administration no topotecan could be detected in plasma during the follow-up period nor in the vitreous of treated and control animals after 1 week of the last injection. The present data shows that four weekly intravitreal injection of 5 μg of topotecan is safe for the rabbit eye. Despite multiple injections of 0.5 μg of topotecan are also safe to the rabbit eye, lactone topotecan vitreous concentrations were potentially active only after 5 h of the administration. We postulate promising translation to clinics for retinoblastoma treatment.

* Corresponding author. Tel.: +54 11 4308 4300x1676; fax: +54 11 4308 5325.
E-mail address: paula.schaiquevich@gmail.com (P. Schaiquevich).

[1] Paula Schaiquevich and Guillermo Bramuglia have equally contributed to the design and development of the work described here.

0014-4835/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.exer.2013.01.002

1. Introduction

Vitreous seeding of tumoral cells constitute one of greatest obstacles to cure intraocular retinoblastoma, so new modalities for local therapy are being explored to improve the drug disposition to the vitreous (Rodriguez-Galindo et al., 2007). Intravitreal administration of chemotherapy drugs for the treatment of retinoblastoma has recently gained significant attention (Munier et al., 2012a,b; Seregard and Singh, 2012). Despite it could be seen as a rather simple technique for drug administration, it carries the risk of orbital dissemination by altering the anatomical integrity of the eye. Orbital tumor seeding through the needle track is a possible serious complication of this technique, however most patients treated by this route did not present orbital relapse and recent techniques to minimize this risk have been published (Munier et al., 2012a,b). In addition, since drug levels in the vitreous can be extremely high and maintained during a long period of time after direct intravitreous injection, the risk of retinal toxicity should be carefully considered and it could limit the use of this technique (Buitrago et al., 2010). Because of these limitations, intravitreous chemotherapy has not became a widespread technique for the treatment of retinoblastoma thus far.

Different drugs have been used by intravitreal injections including melphalan, thiotepa, methotrexate and more recently topotecan (Kaneko and Suzuki, 2003; Kivelä et al., 2011; Seregard and Singh, 2012; Darsova et al., 2011). Topotecan is a topoisomerase I inhibitor with a lactone form with proven activity in retinoblastoma cell lines, animal models and patients (Chantada et al., 2004; Laurie et al., 2005). Several routes of administration have been studied for retinoblastoma treatment including intravenous, periocular, intra-arterial including drug delivery systems, but more limited data are available for intravitreal administration (Buitrago et al., 2010; Carcaboso et al., 2007; Chantada et al., 2010; Nemeth et al., 2011; Schaiquevich et al., 2012).

Our previous results have shown that intravitreal injection of 5 μg of topotecan resulted in a substantial improvement of topotecan bioavailability in the vitreous of rabbit eye when compared to the local periocular route or intravenous administration with the additional advantage of a 200-fold reduction in the administered dose (Buitrago et al., 2010). Moreover, topotecan vitreous concentrations remained above potential pharmacologically active levels (determined from in vitro cytotoxicity studies) for about 16 h post-injection. In addition, we and others found very low levels of topotecan in plasma favoring for potential less systemic-related adverse events (Buitrago et al., 2010; Darsova et al., 2011). Therefore, intravitreal administration is the most efficient route for attaining pharmacologically active vitreous concentrations during a considerable interval of time which is important to target retinoblastoma vitreous seeds. However, information of topotecan toxicity to the eye is scarce. In addition, it is possible that lower doses could reach potentially active levels which would be conceivable less toxic to the eye, however there is no pharmacokinetic study supporting their use.

The present study was performed to evaluate topotecan pharmacokinetics after a single 0.5 μg dose and the toxicological effects of intravitreal injections of two different doses of topotecan in rabbits in order to evaluate the potential translation into the clinics of retinoblastoma treatment. Toxicity was evaluated by means of electrophysiological tests (electroretinography), histological examination, hematologic controls and clinical inspection of the animals.

2. Materials and methods

All experiments adhered the tenets of Hospital Garrahan Institutional Committee for Animal Care and the ARVO Statement for the use of animals in ophthalmic and vision research.

The animals were fed standard laboratory food and allowed free access to water and housed under 12-h light–dark cycles. The study cohort was divided into 2 groups:

1) Pharmacokinetic studies at low topotecan dose.
2) Toxicity studies.

2.1. Pharmacokinetic studies after single topotecan dose

A total of 8 eyes were employed to evaluate topotecan pharmacokinetics after a 0.5 μg single dose intravitreal administration. These animals were analyzed only for pharmacokinetics and no other trauma was exerted to these eyes. Anesthetized animals received an intravitreal injection of 0.1 ml of a 5 μg/ml solution of topotecan prepared in 0.9% saline as previously described by our group (Buitrago et al., 2010). Vitreous humor samples (100 μl) were obtained in the anesthetized animal by aspiration of the posterior eye chamber after drug administration as we previously described (Buitrago et al., 2010). Only one sample was obtained per eye.

2.2. Animals and topotecan administration for toxicity assessment

Adult New Zealand rabbits ($n = 9$), weighting between 1.8 and 2.2 kg, were included in this cohort. Animals in this cohort were assigned to 3 groups, A, B and C ($n = 3$ in each group). Before all intravitreal injections and electrophysiological recordings, pupillary midriasis was induced by 5% phenylephrine hydrochloride and 0.5% tropicamide (Fotorretin, Poen Laboratories, Buenos Aires, Argentina) and 0.5% sterile proparacaine hydrochloride ophthalmic solution (Anestalcon, Alcon Laboratories, Buenos Aires, Argentina) was applied in both eyes for corneal anesthesia. Animals in groups A and B received an intravitreal injection of 0.1 mL topotecan in saline solution at a dose of 5 and 0.5 μg, respectively, into the right eye using a 30-gauge needle attached to a tuberculin syringe. The needle was inserted 2 mm posterior to the limbus and directed toward to the center of the globe until the position was checked by direct visualization with external illumination. The same volume of normal saline was injected into the left eye of each animal and served as control. Group C (control group) received 0.1 mL of saline solution in both eyes. The same procedure was performed every week for 4 weeks with a total of 4 administrations in each eye. Eyes were not punctured for vitreous drugs levels or for other reasons during the experiments. Anterior chamber paracentesis was not done. At the completion of the experiments the rabbits were euthanized by intravenous injection of an overdose of pentobarbital sodium (80 mg/kg body weight), and their eyes were enucleated immediately.

A schematic representation of the present animal studies is represented in Fig. 1.

2.3. Pharmacokinetics of topotecan after multiple injections

In animal groups A, B and C used for testing repeated intravitreal injections of topotecan, venous blood samples (500 μl) were collected from the ear vein in heparinized tubes at baseline (before injection on day 1), 3 h after the first administration, before each topotecan or vehicle intravitreal administration on weeks 2, 3 and 4 and one week after the last administration.

In all cases, two hundred μl of blood were immediately centrifuged and 50 μl of plasma or the same amount of vitreous were treated with 200 μl of cold acid methanol to precipitate the proteins and stabilize topotecan lactone form. Methanolic supernatant extracts was stored at −20 °C until analysis. Topotecan lactone and

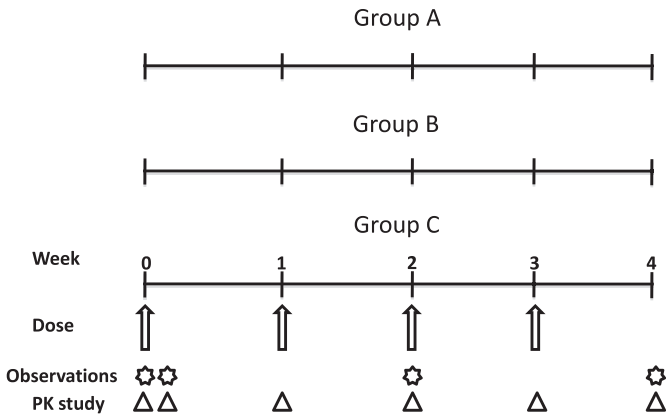

Fig. 1. Treatment plan, clinical and ophthalmological observations after subsequent intravitreal topotecan injections in rabbits. Arrows represent topotecan injections, stars are clinical and ophthalmological observations and triangles, pharmacokinetic study for Group A (0.5 μg/dose), Group B (5 μg/dose) and Group C (vehicle).

carboxylate concentrations in plasma and vitreous samples were determined by HPLC coupled with fluorometric detection as previously described (Buitrago et al., 2010).

2.4. Systemic toxicity evaluation

All animals were examined at weekly basis including weight control, hair loss and general animal conditions. Hematologic values from peripheral blood were determined by an automated flow cytometer (Coulter Counter VCS). The total number of white blood cells, red blood cells, hemoglobin content, neutrophils and platelets were measured. Data was expressed as percentage of neutrophils with respect to total white blood cells and platelet count.

2.5. Ocular toxicity evaluation

Indirect ophthalmoscopic examination was done at baseline (before topotecan injection on day 1) and before each topotecan or vehicle administration in all animals. Electrophysiological recordings (ERG) were carried out in both eyes of each anesthetized animal (ketamine hydrochloride, 37.5 mg/kg, IM and xylazine 5 mg/kg, IM) at baseline, 3 h after the first injection, before the third and one week after the fourth intravitreal administration. The rabbits were dark-adapted for at least 30 min. Each rabbit was placed facing the light stimulus at a distance of 20 cm. A commercial contact lens (ERG-jet®, Fabrinal SA, La Chaux.de.Fonds, Switzerland) with a platinum wire was placed on the cornea as the active electrode while the reference and a grounding electrodes needle were a subcutaneous needle electrode placed on the ear and the occipital crest, respectively. ERG were recorded from each eye and 15 responses to flash of white light (4 ms; 1 Hz) from a photic stimulator (light-emitting diodes) set at maximum brightness (90 cd s/m^2 without a filter), were amplified, then filtered (1.5-Hz low-pass filter; 3000-Hz high-pass filter; notch activated) and averaged (Akonic BIO-PC, Akonic, Buenos Aires, Argentina). The different components of the ERG were evaluated as follows. The a-wave amplitude was measured as the difference in amplitude between the recording at onset and the trough of the negative deflection and the b-wave amplitude was measured as the difference in amplitude between the trough of the a-wave to the peak of the b-wave. Implicit times were also measured and compared between animal groups.

2.6. Histologic examination

All rabbits were euthanatized after ERG recording at 1 week after the last topotecan dose and both eyes were immediately enucleated. The vitreous was separated and stored at −20 C for topotecan quantitation, and each eye was fixed in 4% paraformaldehyde in 0.1 M of phosphate buffer (pH 7.4). The eyes were cut in half and embedded in Paraplast (Leica Biosystems Richmond, Inc, Peterborough, UK). The tissue was cut into 4–5 μm sections. The sections were stained with hematoxylin and eosin. Immunohistochemical study for the expression of glial fibrillary acidic protein (GFAP, Dako, Denmark) in Müller cells was carried out with the use of the streptavidin-biotin- peroxidase technique developed with diaminobenzidine (DAB, Fluka, USA). Histological examination was performed by an experienced pathologist as previously described (Carcaboso et al., 2007).

2.7. Data analysis

Topotecan vitreous concentration-time data after intravitreal injection of 0.5 μg was fit to a compartmental model using the maximum likelihood estimation method as implemented in ADAPT 5 (D'Argenio et al.).

Individual animal weight, hematologic values and ERG data (a- and b-waves and implicit times) was obtained at each time for all animals. Two-way repeated measures analysis of variance (MANOVA) was employed to test for differences between the animal groups (treatment) and also accounting for time as the second dependent variable. We also analyzed the effect of vehicle injection on the retinal function. We compared the retinal function by ERG before any intervention (control eyes) in 12 eyes, corresponding to 3 left eyes of Group A, 3 left eyes of Group B and the 6 eyes of Group C, with respect to the temporal response of those same eyes after weekly injections of saline. This analysis could let us know whether repeated intravitreal injections could affect the retinal function. The evaluations were carried out before vehicle injection, after 3 h, 2 weeks and 1 month of the first injection by means of repeated measure ANOVA with Bonferroni test *a posteriori*. In all cases, the significance level was set at 0.05.

3. Results

3.1. Pharmacokinetic studies

3.1.1. Topotecan vitreous pharmacokinetics after a single intravitreal low dose

Total and lactone topotecan disposition after a single dose of 0.5 μg, was well-described by a two-compartment model as presented in Fig. 2. The model predicted maximum concentration (C_{max}) in the vitreous as lactone and total topotecan was 709 ng/ml and 695.4 ng/ml, respectively (Fig. 2). Vitreous levels above a potentially therapeutic threshold of 14 ng/ml were obtained for lactone topotecan until 5.5 h after intravitreal injection. Lactone and total topotecan vitreous exposure (AUC) was 338.5 ng*h/ml 1096.9 ng*h/ml, respectively.

3.1.2. Topotecan exposure after multiple intravitreal administrations

No topotecan levels after 3 h of the first intravitreal injection or at weekly controls could be detected in any plasma sample from all groups of animals. In addition, topotecan was absent from the vitreous of all studied rabbit eyes after one week of the last administration.

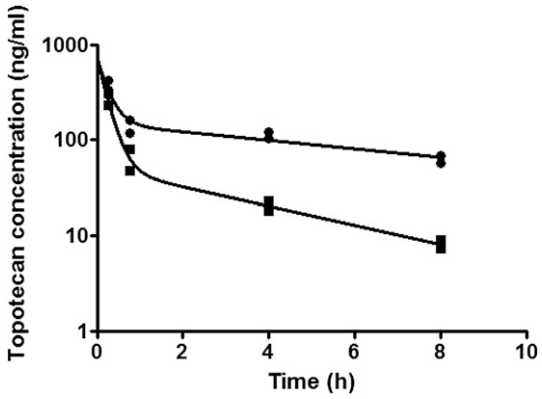

Fig. 2. Total (●) and lactone (■) topotecan concentration *versus* time profile in vitreous humor after intravitreal injection of 0.5 μg. Symbols represent individual data points and lines, the predicted concentrations for total and topotecan lactone in vitreous humor.

3.2. Toxicity evaluation

3.2.1. Clinical observations

Temporal variation of body weight and hematologic values including hematocrit, platelets count, red blood cells count, neutrophils (% with respect to total white blood cells) and hemoglobin content recorded during the studied interval for both topotecan-treated groups and control animals showed no significant differences in any of the evaluated parameter at any period of time as shown in Table 1 ($p > 0.05$). Thus, no significant change in the evaluated parameters could be detected as a result of topotecan treatment (at both doses) or due to temporal variations.

Eyes treated with topotecan (groups A and B) did not show any evidence of inflammation during the follow-up period. The cornea, lens, vitreous and anterior chamber remained clear in all animals. No fundus changes attributable to drug toxicity were evident in any group of animals.

3.2.2. Electrophysiological studies

In order to evaluate the toxicity that could induce the technique of intravitreal injection, we compared the ERGs of eyes before treatment and after weekly injections of saline. We found no significant changes in a-wave, b-wave and implicit times when comparing between groups as shown in Fig. 3 (top row, $p > 0.05$).

As shown in Table 2, we calculated the individual a and b-wave amplitude and implicit time for the topotecan-treated to the control eye of each animal and compared the ERG-ratio parameter among the three studied groups. The time post-treatment was also considered as a dependent variable for the statistical analysis. Data showed similar amplitude and implicit time ranges for all groups at all times before and after serial post-injection with no statistical significant differences as represented in Fig. 3 (low row, $p > 0.05$). All ERGs were normal in all eyes with little or no change in a- and b-waves and implicit times when comparing studied periods of time of animals in the same group and between treatments. Thus, no retinal toxicity according to the ERG responses was found after four weekly doses of intravitreal topotecan throughout the entire follow-up period and until approximately 1 month after the first dose.

3.2.3. Histologic analysis

Retinal sections from topotecan-treated and vehicle-administered eyes were evaluated. Light microscopic examinations revealed no histologic evidence of damage induced by topotecan at any studied dose. Representative micrographs of retinal sections from eyes of rabbits of groups A, B and C are shown in Fig. 4 (top row). In addition, the inner and outer nuclear layers of the retina, the photoreceptor structures and ganglion cells remained normal in animals treated with both topotecan doses. No differences in the retinal thickness were observed between topotecan and vehicle-treated eyes nor with respect to control animals. We observed mild expression of GFAP in scattered Müller cells in the retina of treated and control eyes in the three study groups (Fig. 4, low row).

Table 1
Clinical and biochemical evaluations of the study groups animals after intravitreal administration of 5 μg or 0.5 μg of topotecan or saline.

	Animal group		
	Group A	Group B	Group C
Weight (kg)			
Before treatment	2.6[a]	2.3 (0.1)	2.4 (0.1)
1st week	2.4 (0.2)	2.2 (0.1)	2.5 (0.1)
2nd week	2.4 (0.3)	2.3 (0.1)	2.5 (0.1)
3rd week	2.6 (0.1)	2.4 (0.1)	2.6 (0.1)
4th week	2.5 (0.4)	2.5 (0.2)	2.8 (0.1)
WBC (1000/mm^3)			
Before treatment	5.7[a]	10.0 (4.2)	10.0 (2.7)
1st week	9.8 (3.1)	9.6 (3.9)	8.7 (1.1)
2nd week	8.1 (2.1)	10.4 (5.0)	10.0 (0.9)
3rd week	6.4 (0.8)	7.4 (2.8)	8.9 (0.6)
4th week	6.5 (3.0)	8.1 (5.7)	7.4 (1.7)
Neutrophils (%)			
Before treatment	44,7[a]	47.5 (22.5–65.6	30.6 (20.9–39.3)
1st week	43.0 (42.6–43.3)	40.6 (34.9–46.5	29.4 (18.9–36.1)
2nd week	25.6 (23.0–28.2)	43.9 (32.3–43.7	30.8 (21.4–37.4)
3rd week	29.5 (23.3–41.5)	39.8 (27.7–53.3	33.1 (22.0–45.5)
4th week	37.5 (19.9–62.3)	40.7 (25.8–48.5)	32.1 (25.8–40.7)
Hematocrit (%)			
Before treatment	36.1[a]	47.5 (31.0–42.3)	39.9 (38.1–42.7)
1st week	41.0 (38.2–43.6)	40.6 (38.1–39.0)	40.2 (39.5–42.0)
2nd week	42.9 (37.4–52.6)	43.9 (35.2–42.3)	39.6 (35.8–44.4)
3rd week	40.7 (38.5–42.2)	39.8 (36.5–42.6)	39.9 (38.0–42.5)
4th week	46.2 (44.6–48.2)	40.7 (36.8–43.7)	38.3 (36.7–40.3)
Platelets (/mm^3)			
Before treatment	167,0[a]	206.0 (63.5)	310.3 (48.6)
1st week	263.7 (113.4)	452.0 (101.7)	363.3 (68.8)
2nd week	366.7 (98.1)	423.0 (206.6)	409.7 (110.4)
3rd week	278.0 (16.6)	302.0 (146.9)	262.3 (53.9)
4th week	330.3 (66.5)	398.3 (125.8)	357.3 (96.5)
Hemoglobin (g%)			
Before treatment	11.5[a]	5.7 (8.1)	4.63 (4.1)
1st week	12.9 (1)	6.9 (8.4)	5.45 (3.9)
2nd week	13.4 (2.3)	7.9 (7.7)	6.0 (3.1)
3rd week	12.9 (0.4)	6.7 (8.8)	5.3 (4.3)
4th week	14.6 (0.3)	7.5 (10.1)	6.0 (5.0)
Red blood cells (10^6/mm^3)			
Before treatment	5.6[a]	6.0 (1.1)	6.2 (0.1)
1st week	6.1 (0.49)	6.3 (0.5)	6.5 (0.1)
2nd week	6.3 (1.2)	6.1 (0.9)	6.3 (0.8)
3rd week	6.0 (0.3)	6.1 (0.6)	6.2 (0.3)
4th week	4.6 (3.0)	6.3 (0.6)	5.9 (0.2)

[a] Data was only obtained in one animal due to lost in sampling processing.

4. Discussion

The results from the present study show that intravitreal administration of topotecan up to four weekly injections of 5 μg per dose, did not result in functional or morphologic retinal toxicity in a non-tumor bearing rabbit model. In addition, it did not lead to hematological toxicity or changes in clinical or biochemical parameters potentially related to drug toxicity in our study. There were no differences in toxicity between this dose and that of 0.5 μg,

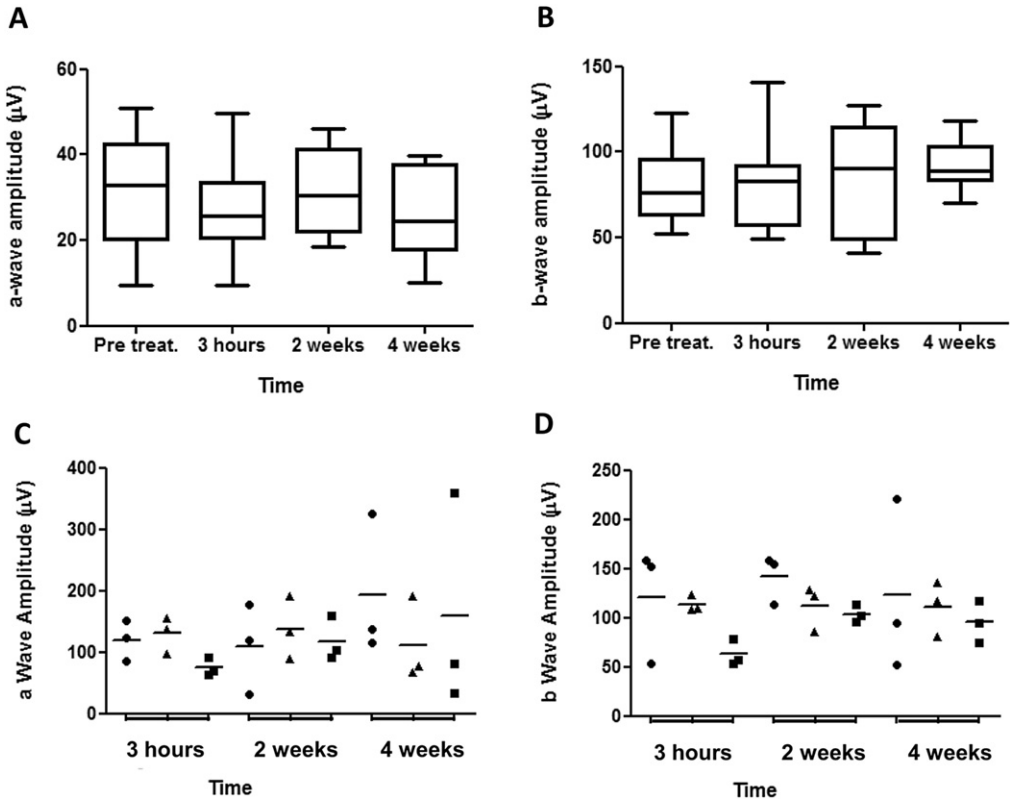

Fig. 3. Boxplot of A, a-wave before and after weekly injections of vehicle ($n = 12$); B, b-wave before and after weekly injections of vehicle ($n = 12$); C, a- wave amplitude after weekly injections of topotecan 0.5 μg (●), 5 μg (▲) or vehicle (■); D, b-wave amplitude after weekly injections of topotecan 0.5 μg (●), 5 μg (▲) or vehicle (■). Horizontal line, median; box, quartiles and whisker, range of the data.

however the latter only achieved potentially therapeutic levels for a shorter period of time.

Topotecan is a potential candidate for use in retinoblastoma because of its activity in preclinical models and children with retinoblastoma (Chantada et al., 2004; Laurie et al., 2005). The major advantages of topotecan, besides its antitumor activity, are based on its relatively low toxicity profile. We have previously reported the vitreous and systemic pharmacokinetics of a single intravitreal injection of 5 μg in the rabbit eye (Buitrago et al., 2010). In that study, we found that this dose led to potentially therapeutic levels that significantly exceed topotecan IC50 for retinoblastoma up to 16 h after the administration. In addition, we observed that topotecan vitreous concentrations during the first hour after drug injection were close to 1 μg/ml. These very high levels with respect to the IC50 and high vitreous exposure even when comparing against any other local route of topotecan administration (periocular, systemic and intra-arterial), could be an advantage for tumor control but it was also a concern for potential toxicity to the retina of those treated eyes (Carcaboso et al., 2007; Schaiquevich et al., 2012). In this sense, we decided to study ocular and systemic toxicity of topotecan after four doses of 5 μg intravitreal injection in a weekly injection to mimic the clinics. According to our data, this treatment is safe as we here report no functional or histologic changes to the retina of the treated eyes or systemic toxicity. In addition, we evaluated topotecan pharmacokinetics and toxicity of a 10-fold lower dose of 0.5 μg (Food and Drug Administration Guidance, 2005). With this dosage, topotecan vitreous exposure measured as maximum concentration was about 700 ng/ml, which is about 10 times lower than the model predicted C_{max} attained after 5 μg in our previous report (Buitrago et al., 2010). However, the area under the vitreous concentration *versus* time profile (AUC) for total (1096.9 ng*h/ml) and lactone (338.5 ng*h/ml) topotecan after 0.5 μg was about 20 times lower than that obtained after 5 μg. Hence, we conclude that topotecan does not follow linear pharmacokinetics in the range of 0.5–5 μg intravitreal administration. This observation may result from a saturation of topotecan transport processes responsible of the drug elimination from the eye through the posterior chamber to the plasma. The exact

Table 2
Amplitudes and implicit times after four doses of 0.5 μg, 5 μg or saline to rabbits.

	Animal group		
	Group A	Group B	Group C
b-wave			
Amplitude			
3 h, 1st dose	120.4 (55.9)	96.7 (56.3)	72.9 (13.5)
1 week, 2nd dose	145.7 (35.7)	128.2 (48.2)	79.9 (37.6)
1 week, 3rd dose	112.6 (68.4)	114.3 (4.8)	106.0 (5.1)
Implicit time			
3 h, 1st dose	114.7 (8.7)	112.2 (0.9)	113.3 (11.7)
1 week, 2nd dose	108.1 (17.3)	116.6 (6.0)	119.8 (3.2)
1 week, 3rd dose	111.7 (14.9)	110.7 (15.0)	113.7 (4.6)
a-wave			
Amplitude			
3 h, 1st dose	103.3 (47.8)	102.5 (51.2)	57.1 (23.4)
1 week, 2nd dose	121.9 (71.7)	128.2 (60.6)	87.7 (28.3)
1 week, 3rd dose	76.5 (57.5)	82.8 (5.6)	76.0 (41.6)
Implicit time			
3 h, 1st dose	86.1 (4.6)	96.7 (27.0)	90.1 (6.1)
1 week, 2nd dose	105.9 (32.1)	116.6 (32.1)	107.3 (31.0)
1 week, 3rd dose	102.0 (6.1)	100.7 (9.2)	108.6 (22.9)

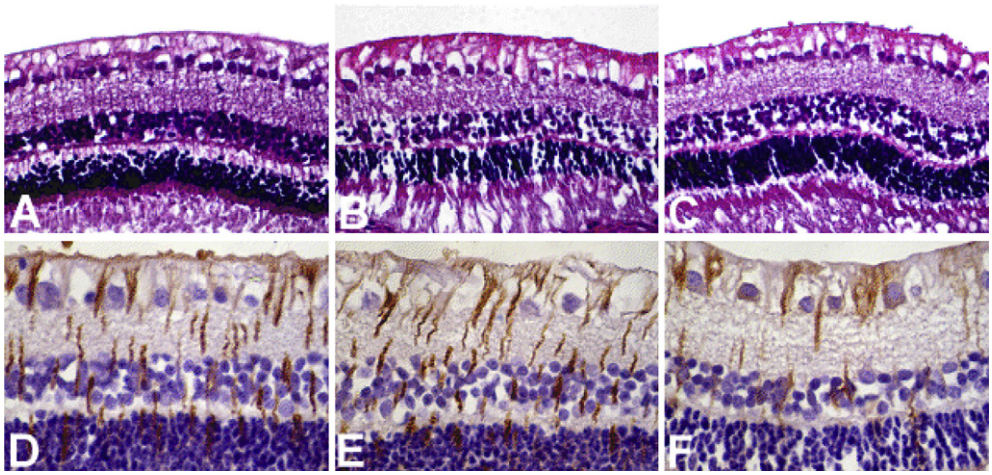

Fig. 4. Upper row, representative micrographs of retinal sections from rabbits of (A) Group A (0.5 μg/dose), (B) Group B (5 μg/dose), and (C) Group C (vehicle) (Objective lens ×40, hematoxilin and eosin stain). Bottom row, GFAP immunostaining of retinal sections from rabbits of (D) Group A (0.5 μg/dose), (E) Group B (5 μg/dose), and (F) Group C (vehicle) (Objective lens ×40).

mechanism for non linear pharmacokinetics should be further studied. When 0.5 μg of topotecan was administered, potentially cytotoxic levels would be reached until 5 h after drug injection which is much less than the exposure obtained with 5 μg that reaches antitumoral effects in the vitreous humor up to 16 h after the intravitreal injection. Repeated administration might lead to drug accumulation in the vitreous compartment depending on the pharmacokinetic characteristics of each drug. However, in the pharmacokinetic study we have previously shown that topotecan half life elimination time from the vitreous was 2.5 h after a 5 μg dose and thus, if the drug is administered in a weekly fashion no accumulation should be expected. Supporting these concepts, we here found no detectable topotecan in the vitreous of all studied animals at the different times after repeated drug administration. Thus, since both dosages were equally non-toxic to the eye, there would not be any advantage of using doses of 0.5 μg in the clinic.

The present results may have an interest for the potential clinical use of intravitreal topotecan for the treatment of retinoblastoma. Intravitreal administration of chemotherapy allows for high drug bioavailability in the vitreous, a situation specifically important in cases of extensive vitreous seeding (Buitrago et al., 2010). Since this route of drug delivery allows for a high local concentration, the major challenge for dose determination is to counterbalance efficacy with drug toxicity to the eye, especially to the retina. In this sense the toxicity of other drugs administered directly through intravitreal injection has been previously studied with the aim of translating their use to patients. Specifically, melphalan is the most extensively studied drug for retinoblastoma treatment. Despite that, there is only one study reporting that perfusion of the vitreous cavity with up to 10 μg of melphalan during vitrectomy without retinal toxicity to the rabbit eye (Shimoda et al., 2008). Based on these results and on previous cytotoxicity studies in retinoblastoma cell lines, Kaneko and Suzuki (2003) incorporated this route of chemotherapy deliver in combination with hyperthermia for synergistic effects with melphalan to the clinics of retinoblastoma patients (Kaneko and Suzuki, 2003). More recently, and continuing the preclinical and clinical experience with intravitreal injections, the most recent reports have shown the experience of multiple intravitreal administration of 20 μg–30 μg per dose of melphalan using a carefully detailed and revised technique of drug administration in retinoblastoma patients. The authors showed excellent results in terms of tumor response and ocular survival at 2 years with minimal ophthalmological adverse events (Munier et al., 2012a,b). Despite limited preclinical information on its toxicity by this route, intravitreal melphalan is becoming used for the treatment of children with high risk retinoblastoma. Intravitreous methotrexate has also been proposed for the treatment of retinoblastoma in a limited number of patients. For this drug, Velez et al. have reported pharmacokinetic and safety data by means of electroretinography and histopathologic examination, after intravitreal administration in rabbits under a chemotherapy treatment that could resemble the clinics of ocular lymphoma (Velez et al., 2001). Indeed, these data has provided the basis for current treatment of intraocular lymphoma. Kivelä et al. (2011) showed methotrexate antitumor activity against retinoblastoma in heavily pretreated eyes receiving such schedule of repeated injections, suggesting that a higher and sustained exposure may be important for tumor control (Chan et al., 1989; Kivelä et al., 2011). Thus, intravitreal chemotherapy delivery is of great potential but information about the disposition of the administered drug, ocular and systemic toxicity becomes essential for optimization of chemotherapy treatment in patients.

In the present study we observed no significant ocular and specifically no retinal toxicity in rabbits after 4 weekly intravitreal injections of up to 5 μg/dose of topotecan in a schedule that resembles the current clinical scheme that is used for other antineoplastic drugs for the treatment of retinoblastoma. This statement is supported by the fact that both rod and cone responses showed no significant changes in the ERG recordings during the studied period after both evaluated topotecan doses compared to control eyes. In addition, we observed no histological changes of the eye structures that could be attributed to topotecan in any of the dosages we studied. We found mild expression of GFAP, an intermediate filament that is normally expressed in astrocytes, in scattered Müller cells which do not express this antigen under normal conditions. Since we found low expression of GFAP in Müller cells of the retinas from rabbits treated with both doses of topotecan but also in control eyes that only received saline, we conclude that it was related to retinal trauma caused by the injection, as previously reported (Woldemussie et al., 2004; Barnett and Osborne, 1995; Shabar et al., 2012).

Our results concurred with those from other groups that evaluated the retinal toxicity of transcorneal topotecan, albeit with a different study design (Darsova et al., 2011). In that study, the authors reported some effects that could also be attributed to trauma to the eye. As opposed to Darsova et al.'s study, in our

present report no eye that was studied for toxicity was punctured for vitreous pharmacokinetics. By doing this, we sought to reproduce the clinical situation where eyes are only injected with drug but not punctured for vitreous pharmacokinetics which may cause severe changes to the vitreous composition and functional alterations in the ocular structures masking any possible effect of chemotherapy induced toxicity. In that sense, Darsova et al. (2011) reported a reduction of ganglion cells with focal retinal atrophy in 2 eyes treated with 1 μg of topotecan but also in 2 vehicle-treated contralateral eyes reported. In addition, these authors observed vitreous hemorrhage, corneal vascularization and lymphocytic infiltration in eyelids in control eyes as well as in few topotecan treated eyes. Despite these histopathologic changes, they showed no statistical significant changes in the different components of the ERG in control or topotecan treated eyes, which are in agreement with our observations. In addition, we observed no evidence of topotecan hematological toxicity with the doses administered, which concurs with the low plasma levels found in our previous studies and others, representing the 1.8% of the total vitreous exposure (Buitrago et al., 2010). Thus, our previous pharmacokinetic data after a single topotecan dose correlates with the present pharmacokinetic analysis and the clinical observations since no hematological toxicity and no hair or weight loss could be detected during the follow-up period.

Even though our data may be useful for translation to the clinical use for retinoblastoma, we acknowledge the following limitations. The rabbit model is extensively used for ocular studies, but anatomical and physiological differences exist between this species and humans. In addition, the presence of tumor disrupts the blood-retinal barrier and may alter chemotherapy vitreous and systemic disposition leading to differences between the present results and those obtained in the clinical setting (Cunha-Vaz, 2004). We here evaluated the functionality of the retinas treated with topotecan by means of ERG after two weeks of the first injection but a possible alteration could have occurred after one week and thereafter resolved. Lastly, the present study was designed for assessing acute toxicity but sub-acute and even chronic toxicity studies after intravitreal administration of topotecan should be carried out. Thus, the present results even if promising should be taken with caution when translating into the patient chronic treatment.

In conclusion, topotecan shows non linear pharmacokinetics after single intravitreal administration of 0.5–5 μg in the rabbit eye. A single 0.5 μg intravitreal dose leads to lactone vitreous levels with potential therapeutic activity after 5 h of drug administration. Lastly, after weekly intravitreal application of up to 5 μg of topotecan, no significant systemic or retinal toxicity according to ERG responses and histological findings was found. No topotecan accumulation was detected in the vitreous after one week of injections.

The present results support the possible translation to clinics of intravitreal topotecan administration for retinoblastoma treatment.

Financial support

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP Nr 11220090100343); Hospital JP Garrahan, Buenos Aires, Argentina; Fund for Ophthalmic Knowledge (GLC, ACF), New York, NY, USA and UBACYT 2011-2014, Nr 20020100100051.

Acknowledgments

The authors want to thank Susana Gorzansany for helping in sample procurement, Gustavo Williams for general assistance with the animal care and Julieta Dominguez for her participation in the ophthalmological evaluation of the animals.

References

Barnett, N.L., Osborne, N.N., 1995. Prolonged bilateral carotid artery occlusion induces electrophysiological and immunohistochemical changes to the rat retina without causing histological damage. Exp. Eye Res. 61, 83–90.
Buitrago, E., Höcht, C., Chantada, G., et al., 2010. Pharmacokinetic analysis of topotecan after intra-vitreal injection. Implications for retinoblastoma treatment. Exp. Eye Res. 91 (1), 9–14.
Carcaboso, A.M., Bramuglia, G.F., Chantada, G.L., et al., 2007. Topotecan vitreous levels after periocular or intravenous delivery in rabbits: an alternative for retinoblastoma chemotherapy. Invest. Ophthalmol. Vis. Sci. 48 (8), 3761–3767.
Chan, H.S., Canton, M.D., Gallie, B.L., 1989. Chemosensitivity and multidrug resistance to antineoplastic drugs in retinoblastoma cell lines. Anticancer Res. 9 (2), 469–474.
Chantada, G.L., Fandiño, A.C., Casak, S.J., Mato, G., Manzitti, J., Schvartzman, E., 2004. Activity of topotecan in retinoblastoma. Ophthalmic Genet. 25 (1), 37–43.
Chantada, G.L., Fandino, A.C., Carcaboso, A.M., et al., 2010. A phase I study of periocular topotecan in children with intraocular retinoblastoma. Invest. Ophthalmol. Vis. Sci. 50 (4), 1492–1496.
Cunha-Vaz, J.G., 2004. The blood–retinal barriers system. Basic concepts and clinical evaluation. Exp. Eye Res. 78 (3), 715–721.
D'Argenio, D., Schumitzky, A., Wang, X. ADAPT 5. Pharmacokinetic/Pharmacodynamic system analysis. User's guide. Biomedical simulation resource, University of Southern California.
Darsova, D., Pochop, P., Uhlik, J., et al., 2011. Topotecan vitreous and plasma levels and retinal toxicity after transcorneal intravitreal delivery in healthy albino rabbits: alternative retinoblastoma treatment. Biomed. Pap. Med. Fac. Univ. Palacky Olomouc, Czech Repub. Dec 20.
Food and Drug Administration Guidance, 2005. Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. http://www.fda.gov/downloads/Drugs/Guidances/UCM078932.pdf.
Kaneko, A., Suzuki, S., 2003. Eye-preservation treatment of retinoblastoma with vitreous seeding. Jpn. J. Clin. Oncol. 33 (12), 601–607.
Kivelä, T., Eskelin, S., Paloheimo, M., 2011. Intravitreal methotrexate for retinoblastoma. Ophthalmology 118 (8), 1689.
Laurie, N.A., Gray, J.K., Zhang, J., et al., 2005. Topotecan combination chemotherapy in two new rodent models of retinoblastoma. Clin. Cancer Res. 11 (20), 7569–7578.
Munier, F.L., Gaillard, M.-C., Balmer, A., Soliman, S., Podilsky, G., Moulin, A.P., Beck-Popovic, M., 2012a. Intravitreal chemotherapy for vitreous disease in retinoblastoma revisited: from prohibition to conditional indications. Br. J. Ophthalmol. 96 (8), 1078–1083.
Munier, F.L., Soliman, S., Moulin, A.P., Gaillard, M.-C., Balmer, A., Beck-Popovic, M., 2012b. Profiling safety of intravitreal injections for retinoblastoma using an anti-reflux procedure and sterilisation of the needle track. Br. J. Ophthalmol. 96 (8), 1084–1087.
Nemeth, K.M., Federico, S., Carcaboso, et al., 2011. Subconjunctival carboplatin and systemic topotecan treatment in preclinical models of retinoblastoma. Cancer 117 (2), 421–434.
Rodriguez-Galindo, C., Chantada, G.L., Haik, B.G., Wilson, M.W., 2007. Treatment of retinoblastoma: current status and future perspectives. Curr. Treat. Opt. Neurol. 9 (4), 294–307.
Schaiquevich, P., Buitrago, E., Ceciliano, A., et al., 2012. Pharmacokinetic analysis of topotecan after superselective ophthalmic artery infusion and periocular administration in a porcine model. Retina 32 (2), 387–395.
Seregard, S., Singh, A.D., 2012. Retinoblastoma: direct chemotherapeutic drug delivery into the vitreous cavity. Br. J. Ophthalmol. 96 (4), 473–474.
Shabar, J., Zemel, E., Perlman, I., Loewenstein, A., 2012. Physiological and toxicological effects of cefuroximeon the albino rebbit retina. Invest. Ophthalmol. Vis. Sci. 53, 906–914.
Shimoda, Y., Hamano, R., Ishihara, K., et al., 2008. Effects of intraocular irrigation with melphalan on rabbit retinas during vitrectomy. Graefes Arch. Clin. Exp. Ophthalmol. 246 (4), 501–508.
Velez, G., Yuan, P., Sung, C., et al., 2001. Pharmacokinetics and toxicity of intravitreal chemotherapy for primary intraocular lymphoma. Arch. Ophthalmol. 119 (10), 1518–1524.
Woldemussie, E., Wijono, M., Ruiz, G., 2004. Müller cell response to laserinduced in intraocular pressure in rats. Glia 47, 109–119.