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Ocular topotecan pharmacokinetics following topical administration to rabbits for diffused anterior retinoblastoma

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Keywords
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Abstract

Objectives We characterized and compared the in-vivo absorption of topotecan into the aqueous humor after instillation of aqueous and ointment formulations.
Methods A lanolin/petrolatum ointment was used. New Zealand rabbits were instilled with topotecan solution (6 μg, group A), a single 10 μg dose of topotecan ointment (group B) or with five 10 μg doses of topotecan ointment (group C). Aqueous humor samples were collected at different times. Corneal samples were collected only for group A. Topotecan was quantified using HPLC, and pharmacokinetic parameters were calculated. Acute corneal epithelial toxicity was assessed after multiple instillations of topotecan ointment.
Key findings Total topotecan maximum aqueous humor concentration (C_{max}) was 16.1, 69.9 and 287 ng/ml in group A, B and C, respectively. A single dose of topotecan ointment increased threefold and sevenfold the aqueous humor C_{max}, and exposure compared to the aqueous formulation. Aqueous humor concentrations from group C eyes were substantially above the cytotoxic concentration for retinoblastoma cells. No corneal toxicity was evident after ointment instillation.
Conclusions Topotecan penetrated into the aqueous humor of the rabbit eye after multiple doses of an ointment in concentrations pharmacologically active against retinoblastoma cells without eliciting acute toxicity. Topotecan ointment may translate to the clinical treatment of anterior segment disseminated retinoblastoma.

Introduction

Retinoblastoma is a tumour that develops in the retina of paediatric patients invading the vitreous space as well as outer ocular structures.[1] Thus, if diagnosed early as usually occurs in developed countries, the tumour is constrained to the state of intraocular disease.[2,3] Nonetheless, malignant cells from the retina invade all directions of the eye globe including the choroid, sclera and all the structures lying in front of the anterior vitreous including the iris, ciliary body, lens, cornea and aqueous humor. Thus, if left untreated, the tumour spreads out of the eye through the optic nerve to the systemic circulation and central nervous system with a consequent associated metastasis and mortality. The latter is the situation of more than half of the children diagnosed of retinoblastoma worldwide who live in developing countries.[4]

In recent years, with the advent of new ocular drug delivery techniques for targeting the posterior segment of intraocular retinoblastoma including super-selective ophthalmic chemotherapy infusion or even the development of safer techniques for intravitreal injection of chemotherapy in eyes with tumours, conservative treatments are intended.[2,5] Moreover, anterior segment invasion was

considered a determinant for enucleation by most clinical groups, but controversy still exists over its value as a risk factor for extraocular dissemination.[6–8] The development of a novel and safe technique for delivering chemotherapy in the anterior segment to target diffuse anterior retinoblastoma has been proposed as part of the development of a retinoblastoma treatment for globe salvage. In two[9] case reports, intracameral injections of chemotherapy allowed control of tumour cells and patients retained the eyes with normal visual function. Despite promising, intracameral injections may lead to complications with emphasis on extraocular seeding of the tumour through the needle track.

Topical therapy for retinoblastoma, to our knowledge, has received little attention before as it has been extensively reported the limited penetration of the drugs to the posterior segment after instillation. However, for aqueous humor seeding targeting, chemotherapy would only need to penetrate the cornea and become available in this anterior fluid.

Topotecan is a well-characterized and widely used chemotherapy agent for retinoblastoma treatment.[10,11] Due to its high solubility in water, it is expected a low penetration of topotecan through the sclera.[12–14] Moreover, instillation of an aqueous solution of a chemotherapeutic agent may spill on cheeks. Thus, to overcome these drawbacks, it would also be interesting to study a dosage form that could increase the viscosity and thus would prevent from spilling, lengthen the time of contact of the drug in the eye and enhance the bioavailability in the aqueous humor.

Altogether, we developed in this study a new formulation of topotecan for topical instillation in the eye to target the anterior chamber in cases of aqueous humor dissemination of retinoblastoma with the ultimate goal of achieving globe salvage. The main advantages of this formulation include the avoidance of injection of chemotherapy with the consequent potential extraocular seeding and the ease of preparation at the pharmacy of a referral hospital in a developing country.

Topotecan is a camptothecin analogue active in small cell lung cancer and metastatic ovarian cancer as well as in several paediatric solid tumours.[15,16] Topotecan in the soluble state undergoes a pH-dependent hydrolysis of the E-ring interconverting the molecule between the active lactone form (in acidic solutions) to an inactive carboxylate form (in neutral and alkaline solutions).[17,18] Moreover, there are several previous reports about the relationship between lactone topotecan systemic exposure and systemic toxicity in paediatric patients with cancer treated with this agent.[19] Therefore, it is of clinical interest to measure both lactone and total topotecan.

In this study, we characterized the in-vivo absorption of topotecan into the aqueous humor after instillation and compare the aqueous humor bioavailability of topotecan following aqueous and ointment formulations of the drug.

Materials and Methods

Test formulations

Aqueous drops of topotecan 0.02% w/v were prepared in laminar flow hood by direct dissolution of commercial topotecan (Topokebir®, Aspen, Argentina) containing 4 mg topotecan, 48 mg mannitol and 20 mg tartaric acid in sterile water and adjusted to pH 6.6 using 0.1 M NaOH. In addition, ophthalmic ointment was prepared by dispersing an aqueous solution of topotecan (0.1% w/v) in an eye ointment base (petrolatum/lanolin, 70 : 30) following the guidelines in USP32 <797>.[20] The resultant ointment of topotecan had a topotecan concentration of 0.033% w/v.

Animals

This work adheres to the tenets of Association for Research in Vision and Ophthalmology for the use of Animals in Ophthalmic and Vision Research and was approved by the animal welfare committee at Hospital de Pediatría JP Garrahan, Argentina.

Twenty New Zealand White rabbits (weighing 1.8–2.3 kg) were evaluated by an ophthalmologist to determine whether they were free of signs of ocular inflammation or gross abnormalities and then were employed in this study. The animals were fed standard laboratory food, given free access to water and housed under 12-h light–dark cycles.

In-vivo *pharmacokinetic study*

Animals were randomly divided into three groups. The first cohort consisting of five animals (10 eyes) received a dose of 6 μg of topotecan to both eyes in a 30 μl drop of the topotecan solution (0.02% w/v of topotecan). This concentration was selected based on previous reports about topotecan stability in aqueous solution at room temperature for 28 days.[21] In the second group, five rabbits (10 eyes) received a single dose of 10 μg of drug in 30 μl of the topotecan ointment (0.033% w/v of topotecan) into both eyes through a sterile syringe. In this case, we selected the highest dose of topotecan that could be loaded in the ointment according to the water loading capacity of the lanonine/petrolatum combination. The bilateral administration strategy was employed to reduce the number of animals employed in the study based on 3R principles (reduce, refine and replace) as previously detailed elsewhere.[22] Lastly, the third group consisted of eight rabbits (16 eyes) and was allocated to the multiple-dose pharmacokinetic study. Therefore, these eyes received five doses of 10 μg of

topotecan in 30 μl of the topotecan ointment every 2 h. In all groups of animals, we gently pulled the lower lid to form a cup, and the drug was instilled in the cul-de-sac of the eye. Then, the upper and lower lids were held closed for 10 s to minimize drug loss.

Before aqueous humor sampling, rabbits were anesthetized with ketamine (30 mg/kg, IM; Fada Pharma, Buenos Aires, Argentina) and xylazine (2 mg/kg, IM; Calier, Buenos Aires, Argentina). In addition, local topical anaesthesia consisting of 0.5% proparacaine hydrochloride ophthalmic solution (Anestalcon; Alcon Laboratories, Buenos Aires, Argentina) was applied to the cornea. Pupillary mydriasis was induced using 5% phenylephrine hydrochloride and 0.5% tropicamide (Poen, Buenos Aires, Argentina) to avoid lacerating the iris during aqueous humor sampling. Aqueous humor was sampled using a 31-gauge needle after 5, 15, 30, 45 and 60 min after drops instillation; 0.5, 1, 2, 4 and 6 h after ointment instillation for the single dose; and 0.5, 1, 2, 3 and 4 h after the last instillation for the multiple-dose pharmacokinetic study. In all cases, only one sample was collected from each eye to preserve the ocular physiology.

At the end of the study, all animals were euthanized under anaesthesia with a KCl (5 meq/kg) bolus injection. Only in the group that received the drops, the eye was enucleated and the cornea separated for topotecan assay. Only one sample from the aqueous humor and cornea was obtained from each eye.

Sample processing and topotecan analytical assay

Topotecan hydrochloride analytical standard (99% purity) was donated by Asofarma (Argentina). All solvents were high-pressure liquid chromatography (HPLC) grade (Sintorgan, Buenos Aires, Argentina). HPLC grade water was obtained using a Milli-Q system (Millipore Corporation, Billerica, MA, USA).

Both lactone and total topotecan were assayed in the methanolic supernatants using a previously validated analytical technique.[23]

Briefly, the analysis was performed with an Agilent (Santa Clara, CA, US) HPLC system equipped with an Agilent 1100 liquid chromatography pump and an Agilent fluorescence detector set at an excitation/emission wavelength of 370 and 530 nm, respectively. Separation chromatography was performed using a Nova-pack C18 reverse-phase column (150 × 3.9 mm, 4 μm particle size; Waters, Milford, MA, US.) coupled to a C18 Phenomenex security guard precolumn. The mobile phase consisted of acetonitrile–triethylamine buffer pH = 5.5 (85 : 15, v/v) delivered at a flow rate of 1.0 ml/min, and the injection volume was 20 μl. Data acquisition and processing were performed using the Agilent ChemStation software. Stocks solutions of topotecan were prepared in methanol and stored at −20°C to minimize degradation.

The calibration curves were prepared over the concentration range 1–500 ng/ml by dilutions of stock solutions with PBS standards and thereafter processed similar to the real samples. The lower limit of quantitation was 1 ng/ml. Interday precision was <14% for topotecan in methanolic extracts.

After aqueous humor removal, 50 μl of each sample was mixed with 50 μl of methanol for lactone topotecan or with 50 μl of acidic methanol (methanol/HCl, 10 : 1) for total topotecan, vortexed for 10 s and centrifuged at 105000 g for 2 min, and the supernatant was storage at −20 °C until HPLC analysis.

Corneas from animals treated with the topotecan aqueous solution were weighed and homogenized with cold acidic methanol (1 mg of tissue : 4 μl acidic methanol) using IKA T25 Ultra-Turrax. Despite it would have been desirable to measure topotecan in the cornea of the animals after ointment instillation; due to the properties of the ointment, it was difficult to separate the ointment from the cornea. It could be obtained a fictitious result because of the contamination. Then, we only focus in the aqueous humor concentration that is the main target for cell dissemination in diffuse infiltrating retinoblastoma.[24]

Pharmacokinetic analysis

A one-compartment model was fit to topotecan aqueous concentration vs time data using the maximum-likelihood estimation method implemented in ADAPT V.[25] The model was parameterized in terms of a first-order apparent elimination rate constant (k_{el}) and apparent absorption rate constant (k_a), and the volume of distribution in the aqueous compartment was fixed to 0.2 ml based on previous reports.[26] Data from all animals belonging to the same group were pulled together for a naïve pooled-data approach. Estimated pharmacokinetic parameters of aqueous humor after single-dose instillation of topotecan ointment were used to simulate the concentration vs time profile that would be obtained in the steady state after different intervals of ointment administration. For instance, we simulated the steady-state concentrations attained if administering the ointment every 1, 2 or 4 h. Based on the results obtained by the simulations, we decided the scheme for the pharmacokinetic study after multiple-dose administration.

Finally, estimated pharmacokinetic parameters of topotecan were used to simulate the aqueous humor concentration vs time curve from which the area under the curve up to the last measurable concentration (AUC) was calculated using the linear trapezoidal method. To estimate

the total AUC (AUC_{total}), the area beyond the last observed concentration was estimated by dividing the last detectable concentration by the terminal elimination rate constant.

Ocular toxicity

Acute toxicity of topotecan ointment instillation was evaluated in a separate cohort of two animals (four eyes). The eyes were examined the day before and 1 h after the last dose of ointment for corneal and conjunctiva abrasion using a hand-held slit-lamp and fluorescein test (Fotoptic®; Poen).

Results

After the administration of the aqueous solution of topotecan, total topotecan was detected in the aqueous humor only at 45 and 60 min showing mean values of 9.1 and 16.1 ng/ml, respectively. Thus, topotecan total AUC was only 6.2 ng h/ml (Table 1). No topotecan in the lactone form was detected in the aqueous humor. On the contrary, topotecan was substantially detected in the cornea of all the studied eyes after instillation of the aqueous solution achieving a mean maximum concentration (C_{max}) of 0.13 ng/mg tissue after 5 min of the administration.

After a single-dose topical administration of the ointment, a total topotecan aqueous humor C_{max} of 69.9 ng/ml was observed attained after 2 h of the administration (Table 1). The data were adequately fitted to a one-compartment model as shown in Figure 1a. In addition, lactone topotecan aqueous C_{max} was 11 ng/ml after only 0.5 h and thereafter levels declined fast as shown in Figure 1b. A rapid elimination of topotecan as the lactone form was observed, and only 4 ng/ml could be detected after 4 h of ointment instillation. Moreover, the calculated lactone and total topotecan AUC were 34.6 and 122.6 ng h/ml, respectively. Moreover, the extrapolated AUC to infinity (AUC_{total}) was 37.3 and 138.2 ng h/ml for lactone and topotecan total aqueous humor exposure, respectively. Thus, the sampling time interval was adequate as more than 80% of the AUC_{total} corresponded to the estimated AUC up to the last observation at 6 h after instillation.

The elimination rate constant of total topotecan after a single dose of the ointment was 0.32/h, and thus, the elimination half-life $T_{1/2}$ of 2.2 h was based on a one-compartment model estimation ($k_{el} = \ln(2)/T_{1/2}$).[27] For the lactone, kel was 0.48/h and $T_{1/2}$ of 1.5 h. To attain topotecan accumulation in the aqueous compartment, the frequency of ointment instillation should be less than or equal to the half-life time in this compartment thereby selecting an interval of instillation of 2 h for the multiple-dose study. Based on the pharmacokinetic parameters obtained for total and lactone topotecan in aqueous

Table 1 Topotecan pharmacokinetic parameters after single and multiple instillation of drops and ointment

Estimated pharmacokinetic parameter of aqueous humor	Aqueous drops	Topotecan ointment	
		Single dose	Multiple dose
C_{max} (ng/ml)	16.1	69.9	286.7
C_{max}/D (ng/ml)/μg	2.7	7.0	28.7
AUC (ng h/ml)	6.2	122.6	431.7
AUC/D (ng h/ml)/μg	1.0	12.3	43.2
AUC_t (ng h/ml)	–	138.2	–

AUC, area under the concentration vs time up to the last observed concentration; AUC_t, area under the concentration vs time extrapolated to infinity; C_{max}, maximum total topotecan aqueous humor concentration; D, dose.

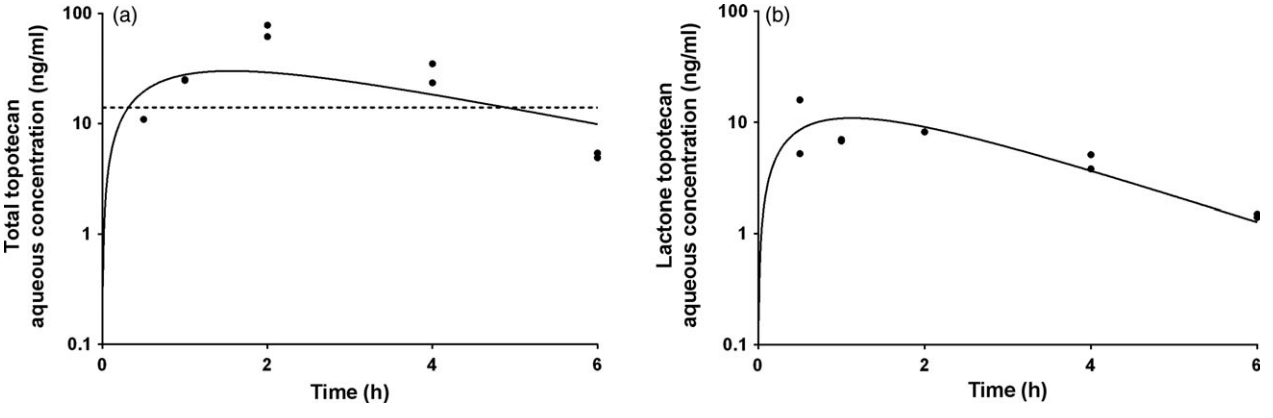

Figure 1 Concentration vs time profile of aqueous humor topotecan after single-dose administration of 0.033% topotecan ointment to rabbits. Aqueous humor total (a) and lactone (b) topotecan concentration vs time profile after a single-dose administration of 30 μl ointment (topotecan 0.033% w/v) to rabbits. Symbols represent the observed concentration, and the continuous line, the best-predicted concentrations.

humor, we performed simulations to establish the most convenient interval of ointment administration to exceed and maintained prolonged pharmacologically active concentrations in this compartment. In this sense, we assumed the active concentration as the one that inhibits the proliferation of 50% of retinoblastoma cells in vitro (IC_{50}), previously determined as 30 nM equivalent to 14 ng/ml.[11] Besides, we also took into account the dose interval that would be technically feasible to propose the multiple-dose study.

Lastly, in a separate cohort of animals, the instillation of five doses of 30 µl of ointment allowed attaining a total topotecan aqueous humor C_{max} of 286.9 ng/ml after 1 h of the fifth dose as shown in Figure 2a, whereas the lactone topotecan C_{max} was 36.7 ng/ml at 1 h as depicted in Figure 2b. Remarkably, total topotecan concentrations in the aqueous compartment remained above the IC_{50} for at least 4 h after the last instillation. Finally, the calculated lactone and topotecan total AUC were 92.1 and 431.7 ng h/ml, respectively, as depicted in Table 1.

Finally, as shown in Figure 3, no evidence of corneal epithelial abnormalities was observed after five doses of topotecan ointment proving the lack of acute toxicity of the developed ointment.

Discussion

This study was designed to assess the bioavailability in the aqueous humor of topotecan after the instillation of an aqueous solution as drops compared to an ointment loaded with the drug. After topical instillation of a single dose of topotecan ointment into the rabbit's eye, a threefold increase in the maximum concentration and a sevenfold increase in the area under the concentration vs time profile of topotecan in aqueous humor were observed compared to the concentration attained after topotecan solution instillation. Moreover, after instillation of multiple doses of topotecan ointment, topotecan concentrations in the aqueous humor were above the pharmacologically active

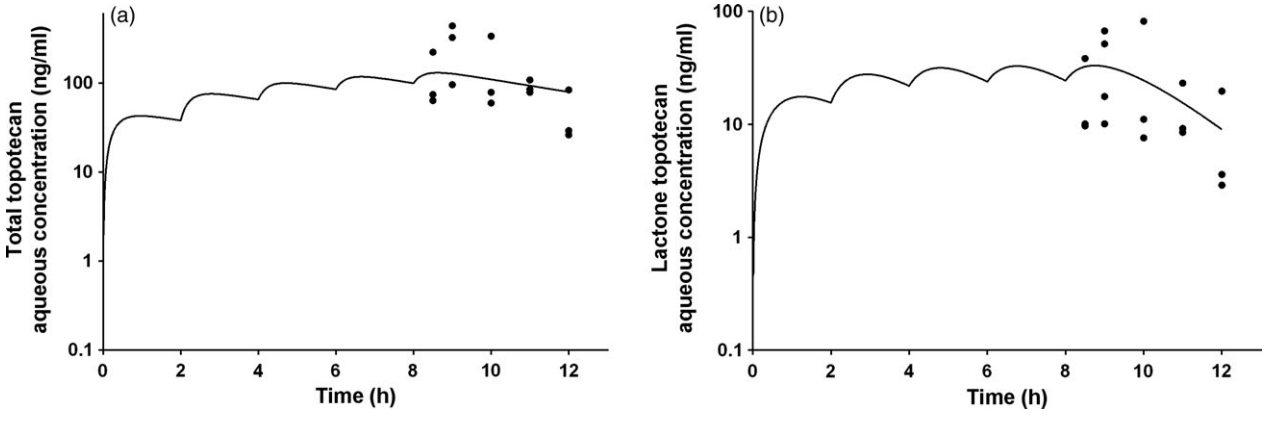

Figure 2 Topotecan concentration in the aqueous humor after multiple instillation of 0.033% topotecan ointment. Aqueous humor total (a) and lactone (b) topotecan concentration vs time profile after multiple instillation of 30 µl ointment (topotecan 0.03% w/v) to rabbits. Symbols represent the observed concentration, and the line, the best-predicted concentrations. The dotted line (a) shows the previous predicted IC_{50} of 14 ng/ml.[11]

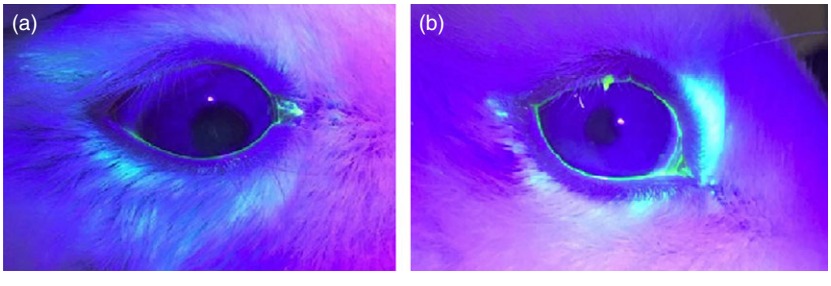

Figure 3 Corneal evaluation with slit-lamp biomicroscopy and fluorescein staining as a measure of ocular surface toxicity. The figure shows a representative rabbit eye (a) before and (b) after multiple topotecan ointment instillations with fluorescein staining. No changes in corneal epithelial were observed after ointment instillation. [Colour figure can be viewed at wileyonlinelibrary.com]

concentration established *in vitro* in retinoblastoma cell cultures for at least 4 h.

The cornea has been extensively described as a barrier for drugs to achieve the aqueous humor after instillations.[28,29] To be effective, antineoplastic drugs must penetrate across the cornea to attain the aqueous humor where retinoblastoma cells are seeded either at diagnosis or especially after failure of conservative therapy.[6] Thus, for a potential clinical use, it is necessary to maximize the transcorneal penetration and determine the concentrations attained in the aqueous humor by means of pharmacokinetic studies to assess whether the anterior route is suitable for topotecan delivery to the aqueous humor. To prevent the rapid elimination by the drainage system of the eye, the administration of more viscous solutions prolongs the contact of the drug to the cornea and thus improves the bioavailability in the aqueous humor by increasing the penetration through the cornea.[30,31] Lanolin is well-known for increasing drug penetration in dermic administration.[32] Thus, it was reasonable to expect an in-vivo increase in the penetration of topotecan after ointment instillation compared to the instillation of the aqueous solution. In addition, lanolin/petrolatum is a safe combination used for instillation in patients with eye dryness as artificial tears. Besides, an advantage of its use is its ease preparation at a hospital pharmacy. Therefore, we tested this formulation for preparing and characterizing topotecan ointment.

An important therapeutic benefit of an instilled formulation is the potential of translation into the clinical practice as patients with retinoblastoma with anterior chamber seeding would not need to be subjected to repetitive intracameral injections for attaining therapeutic levels of chemotherapy.[9] Moreover, another advantage of the topical delivery is related to reducing ocular pain and discomfort and importantly, avoids potential extraocular seeds in the needle track after injection and thus is a significant clinical advancement. Interestingly, there are no commercial topotecan eye drops or other formulations for retinoblastoma treatment. Thus, the present is the first formulation developed with the aim of studying the anterior route of drug delivery for this disease in children.

In aqueous solution and biological fluids, topotecan undergoes a pH-dependent hydrolysis interconverting the molecule between the active lactone and the inactive carboxylate form.[17,18] Even though the active form is the lactone topotecan, the reported IC_{50} in commercial retinoblastoma cell lines is 14 ng/ml of total topotecan.[11] Therefore, this concentration was pursued in this study for the aqueous humor concentrations. In this study, we showed that after topical dosing of a single dose of topotecan ointment into the rabbit eye, the maximum aqueous humor concentration was reached at 2 h and the maximum concentration in the aqueous humor notably exceeded those attained after instillation of the aqueous solution (Figure 1). Furthermore, aqueous humor topotecan concentrations after instillation of the ointment were above the pharmacologically expected threshold far longer than after instillation of the aqueous solution of topotecan that only showed a sole measurable concentration with a fast elimination from the aqueous compartment. One explanation could be the increase in topotecan cornea contact enhancing the penetration to the aqueous humor of the animals, but the exact mechanism should be further explored. Based on these results, we used estimated pharmacokinetic parameters of topotecan aqueous humor to perform simulations to predict the concentrations that would be obtained after multiple instillations of ointment. We simulated the aqueous humor concentrations that would be attained if administering the ointment every 1, 2 or 4 h. The results showed that if the ointment would have been instilled every 4 h, there would not be enough accumulation of topotecan in the aqueous humor as the interval of dosing was greater than the elimination half-life (2.2 h). Despite the scheme of instillation of every hour leads to simulated topotecan concentrations far above the pharmacologically active levels expected for the aqueous humor, this frequency of instillation would not be feasible to be performed in the clinics. If topotecan ointment was instilled at a frequency equal to the elimination half-life, the maximum concentration attained at steady state would be twice the C_{max} during the first dosing interval. Thus, based on feasibility and also on the pharmacokinetic approach, we decided to perform the multiple-dose pharmacokinetic study using a frequency of instillation every 2 h.

In a different cohort of rabbits, we showed that after multiple administration of topotecan ointment, the aqueous humor concentrations were well above the IC_{50} for at least 4 h after instillation. Interestingly, topotecan total exposure in the aqueous humor was greatly enhanced after ointment instillation as showed by the 12-fold increase in the AUC compared to the instillation of the aqueous solution in the present animal model. Moreover, there were no corneal epithelial changes after multiple doses of topotecan ointment supporting the lack of acute toxicity and potential clinical translation of the formulation.

One limitation of this study is that multiple doses of topotecan loaded in the ointment are needed to achieve pharmacologically active concentrations of the drug in the aqueous humor. However, a treatment regimen of multiple administrations is inconvenient for an outpatient setting and taking into consideration that topotecan can be

absorbed through the skin. To comply with animal welfare international guidelines, we instilled local anaesthesia that could alter the pharmacokinetics of the studied drug. Nonetheless, the same topical anaesthetic is currently used in the clinics of our patients during ocular procedures. Despite promising, the present results should be translated into the clinics with caution as further preclinical toxicity studies should be carried out to assess for potential alterations to the cornea after multiple instillation of topotecan ointment.

Altogether, we developed an easy and feasible topotecan ointment prepared in the pharmacy at a clinical centre. Topotecan can penetrate to the anterior chamber into the aqueous humor of the rabbit after single- and multiple-dose administration of an ointment in concentrations that could be pharmacologically active against retinoblastoma cells. This formulation may have utility in treatment of diffuse anterior retinoblastoma.

Declarations

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