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ORIGINAL ARTICLE

Pharmacokinetics of Melphalan After Intravitreal Injection in a Rabbit Model

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Abstract

Purpose: Although widely used for vitreous seed control in retinoblastoma patients, currently there are no data on melphalan pharmacokinetics after intravitreal injections. Therefore, in this study, we characterized the ocular and systemic disposition of melphalan after intravitreal injection in the rabbit eye.
Methods: New Zealand rabbits received a single intravitreal injection of 15 μg of melphalan. Vitreous, aqueous, retina, and blood samples were collected at different times up to 12 h after the injection. Melphalan was quantitated in the biological samples using a validated high-performance liquid-chromatography technique and pharmacokinetic parameters were calculated by means of compartmental models.
Results: Model-predicted melphalan maximum vitreous, aqueous, and retina concentrations were 7.8 μg/mL, 0.024 μg/mL, and 9.8 μg/g tissue, respectively, attained immediately and at 0.8 and 0.25 h after intravitreal injection. Melphalan vitreous concentrations were higher than 0.3 μg/mL for 5 h after dosing. The elimination half-life from the vitreous, aqueous humor, and retina was 1.0, 0.2, and 1.2 h, respectively. Aqueous exposure [area under the curve (AUC)] was only 0.7% of that of the vitreous AUC. Melphalan concentrations in the retina were still detectable 12 h after dosing, while plasma exposure was under the limit of quantitation.
Conclusion: Intravitreal administration of 15 μg melphalan leads to pharmacological vitreous levels with low aqueous exposure. Melphalan concentrations in the retina were measurable up to 12 h after dosing, but we report nondetectable systemic exposure in the rabbit. The results correlate with the clinical features of retinoblastoma patients that show control of vitreous seeds without systemic toxicity using intravitreal melphalan.

Introduction

Retinoblastoma is the most common primary intraocular cancer in children.[1,2] Historically, response rates of eyes with vitreous seeds treated with systemic chemotherapy and focal therapy were low and most eyes required enucleation. Less than a decade ago super-selective ophthalmic artery infusion and a novel safe technique for intravitreal injection of chemotherapy were introduced into the clinical practice changing the landscape of retinoblastoma treatment and ocular outcome.[1,2] Specifically, the intravitreal injection of drugs has proven to be an efficacious technique for treating eyes with recurrent vitreous seeding, improving the prognosis for eye preservation even in eyes with advanced retinoblastoma (group D, International Classification).[3,4] This route of drug delivery allows attaining high pharmacologically active levels in the vitreous for prolonged periods of time.[5,6] The increased bioavailability of chemotherapy in the vitreous is the reason for choosing the intravitreal injection route for targeting the vitreous seeds, which are still the major cause of failure of conservative therapy in retinoblastoma.[3,4,7,9–11] Initially ruled out because of the possibility of orbital seeding, intravitreal injections of melphalan using an improved injection technique have been incorporated into the therapeutic management of retinoblastoma with successful vitreous seed control.[3,11,13,14]

Based on sensitivity assays in retinoblastoma cell lines carried out by Inomata and Kaneko, the most widely used agent for targeting vitreous seeds is melphalan; however, currently no data on ocular disposition are available.[15] The authors proposed that a 10-μg intravitreal injection to the rabbit eye results in a vitreous concentration that is pharmacologically

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active against retinoblastoma cells. In addition, retinal function and structure after intravitreal injection with melphalan were assessed by Ueda et al. in albino rabbits.[16] After a dose of 10 μg, the retina remained unaltered, but severe changes were reported by the authors after doubling the dose to 20 μg. In agreement with these results, in a previous study, we showed histopathological evidence of retinal damage and a decrease in the electroretinogram response as a surrogate for retinal toxicity in pigmented rabbits after repeated intravitreal injections of 15 μg of melphalan.[17] In the latter study, the observed retinal impairment may have been related to an accumulation of melphalan in the retinal tissue. Nonetheless, there are no previous reports on the disposition of melphalan in the eye after intravitreal injection, despite well-documented clinical outcomes in patients with retinoblastoma.

Therefore, the aim of this study was to assess the ocular and systemic disposition of melphalan in the rabbit after a single intravitreal injection of a dose related to the current clinical practice and adapted to the size of the rabbit eye. In addition, we determined the time to achieve a reduction of the viability of an *in vitro* culture of a retinoblastoma cell line and the relationship with the *in vivo* ocular disposition characterized in rabbits.

Methods

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.

Melphalan administration and sampling schedule

Fourteen New Zealand White rabbits (weighing 1.8–2.3 kg) were employed in this study. The animals were fed standard laboratory food, given free access to water, and housed under 12-h light–12-h light dark cycles. For the intravitreal injection, rabbits were anesthetized with ketamine (37.5 mg/kg, IM; Fada Pharma) and xylazine (5 mg/kg, IM; Calier), and corneal anesthesia was obtained after the instillation of 0.5% proparacaine hydrochloride ophthalmic solution (Anestalcon; Alcon Laboratories). Pupillary midriasis was induced using 5% phenylephrine hydrochloride and 0.5% tropicamide (Poen).

Each animal received an intravitreal injection of 15 μg of melphalan dissolved in 0.05 mL of 0.9% saline to both eyes using a 31G 5/16″ gauge needle (BD® insulin syringe, catalog No. 328440). The needle was inserted superotemporally, 5 mm from the limbus and directed toward the center of the globe. Anterior chamber paracentesis was not performed. The selected dose was based on our previous studies on the toxicity of melphalan after intravitreal injection in an animal model and patients with retinoblastoma.[17] In that study, patients received 30 μg of melphalan intravitreally. Considering a human vitreous volume of 3.5 mL and scaling to the rabbit's vitreous volume (1.5–2.0 mL) yielded an equivalent dose between 13 and 17 μg.[18] The current clinical dose used by Suzuki et al. in one of the largest series of patients is between 16 and 24 μg depending on tumor burden.[12] Therefore, we chose a dose of 15 μg for the previously published toxicity assessment and the present pharmacokinetic study.

To minimize the number of animals based on 3R principles (Reduce, Refine, and Replace), we first tested a pilot group of 4 animals ($n = 4$) showing that no melphalan could be detected in the nontreated fellow eyes of the intravitreally injected animals.[18] In these first 4 rabbits, vitreous humor, aqueous humor, and retina samples from both eyes were obtained as previously described 1, 2, 6, and 12 h after intravitreal injection. Melphalan could not be detected in the retina or the vitreous of the contralateral (nontreated) eye of the 4 animals at any time after the injection to the right eye, and therefore, all subsequent animals were injected with melphalan to both eyes so as to reduce the number of animals employed in the study. To obtain enough data for robust estimates of the pharmacokinetic parameters of melphalan after intravitreal injection, we collected samples at 8 different times after dosing with at least 3 replicate eyes used for each time point. Thus, a total of 24 eyes from 14 animals were used to characterize melphalan pharmacokinetics after intravitreal injection. Vitreous humor samples for the evaluation of drug levels were obtained by aspiration at 0.083, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 12.0 h. For every 100 μL of vitreous samples, 110 μL of cold methanol/HCl (10:1) was added. Afterward, the rabbits were sacrificed using an intravenous injection of an overdose of pentobarbital sodium (80 mg/kg body weight), and their eyes were immediately enucleated. Thereafter, the retinas were dissected, washed with cold phosphate-buffered saline, weighed, and manually homogenized with methanol/HCl solution in a 1-to-5 dilution. Methanolic supernatants were stored at −20°C until melphalan assay. In all cases, only 1 sample was collected from each eye to preserve the ocular physiology.

Venous blood samples (0.2 mL) were collected from the ear vein in heparinized tubes at the same times that vitreous humor samples were obtained, but after only 1 eye had received the intravitreal injection of melphalan.

Reagents and melphalan analytical assay

Melphalan hydrochloride was purchased from Sigma-Aldrich. Stock solutions of melphalan were prepared in methanol and stored at −20°C to minimize degradation. Solvents were high-pressure liquid chromatography (HPLC) grade (Sintorgan). HPLC-grade water was obtained using a Milli-Q system (Millipore Corporation).

Melphalan concentrations were determined by HPLC coupled with a fluorescence detector according to a method previously developed and validated by our group.[19] Briefly, the analysis was performed with an Agilent HPLC system equipped with an Agilent 1100 liquid chromatography pump and an Agilent fluorescence detector set at an excitation/emission wavelength of 270 and 350 nm, respectively. Separation chromatography was performed using a Nova-pack C18 reverse-phase column (150 × 3.9 mm i.d., 4 μm particle size; Waters) coupled to a C18 Phenomenex security guard precolumn. Data acquisition and processing were performed using the Agilent ChemStation software. The linear ranges for plasma and vitreous/aqueous humor assays were from 0.01 to 0.70 μg/mL and from 0.01 to 10.0 μg/mL, respectively. Interday precision was <11% for melphalan in methanolic extracts and vitreous except for the lowest concentration that had an interday precision of <16%.

Pharmacokinetic analysis

Data from all animals were pulled together for a naive pooled-data approach and a 1-compartment model was fit to melphalan vitreous concentration-time levels using the maximum

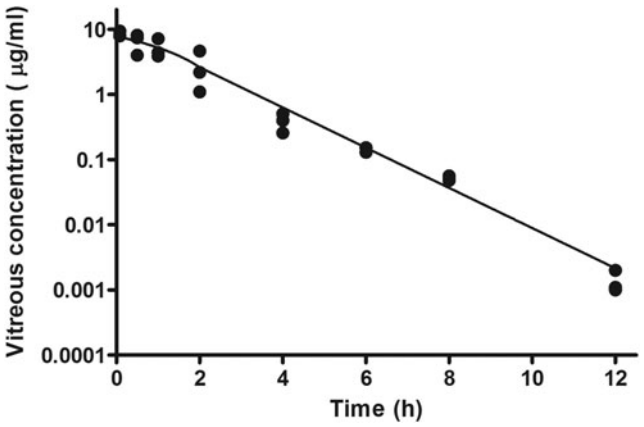

FIG. 1. Melphalan pharmacokinetics in the vitreous after intravitreal injection of 15 µg in the rabbit. Symbols represent individual data points and the *solid lines* show the best-predicted concentrations ($n = 3$ observations per time point).

likelihood estimation method (ADAPT II).[20] The model was parameterized in terms of a first-order elimination rate constant ($K_{el,vit}$) and volume of distribution in the vitreous compartment (V_{vit}).

A 1-compartment model was also fitted to the retina and the aqueous data parameterized in terms of an apparent absorption rate constant ($k_{a,aq}$; $k_{a,ret}$) from the vitreous as the absorption compartment and distributed into the apparent volume of distribution of the retina (V_{ret}/F_{ret}) and aqueous humor (V_{aq}/F_{aq}). The bioavailability factor F in the aqueous and retina compartments is denoted as F, while for vitreous it was assumed as 1. Vitreous, aqueous humor, and retina melphalan-estimated pharmacokinetic parameters were used to simulate the plasma concentration versus time curve from which the area under the curve up to the last measurable time point (AUC_{last}) was calculated using the trapezoidal method.

Results

A total of 24 samples of vitreous, plasma, and retina were obtained in this study for pharmacokinetic analysis.

Interestingly, the disposition of melphalan in the vitreous humor after a single dose of 15 µg was described by mixed-order pharmacokinetics. The concentration versus time data during the first 2 h after injection was described using a zero-order elimination rate process and thereafter the disposition was followed by a first-order process as shown in Fig. 1. The model-predicted maximum concentration ($C_{max,vit}$) of melphalan in the vitreous was 7.8 µg/mL, attained immediately after the intravitreal injection. In addition, the calculated vitreous volume of distribution was $V_{vit} = 1.9$ mL, while the first-order elimination half-life of melphalan from the vitreous was 0.98 h described in Table 1. In addition, vitreous exposure expressed as the area under the concentration versus time profile (AUC_{vit}) was 14.03 µg * h/mL (Table 1). As shown in Fig. 1, the model-predicted vitreous concentration after 1 and 5 h of dosing was 4.7 and 0.3 µg/mL, respectively. Twelve hours after injection, no melphalan could be detected in the vitreous (limit of quantitation of the analytical technique: 10 ng/mL).

Melphalan aqueous humor concentrations were quantifiable between 0.5 and 4.0 h after the injection as only 1 of 3 samples was quantifiable at 6 h and the concentration of that sample was at the limit of quantitation of the analytical technique. Thereafter, the elimination from the aqueous compartment precluded the measurement of detectable levels in the anterior chamber. The elimination half-life of melphalan from the aqueous compartment of 0.19 h was faster than the elimination process from the vitreous. As depicted in Fig. 2 and detailed in Table 1, aqueous exposure of melphalan was only 0.7% of the vitreous exposure. Furthermore, the model predicted C_{max} in the aqueous humor was ($C_{max,aq}$) 0.024 µg/mL attained 0.8 h after injection.

Interestingly, retinal exposure to melphalan was already detected 5 min after and remained detectable up to 12 h after drug administration. Specifically, melphalan exposure parameters (C_{max} and AUC) in the retina are expressed with respect to the weight of the isolated retina in each case as detailed in Table 1. The half-life of elimination from the retina was 1.2 h, close to that from the vitreous. Of note was that although melphalan concentration was below the limit of quantitation in the vitreous 12 h after the single 15-µg injection, the drug was quantifiable in the retina as shown in Fig. 3.

Altogether, the parameters of melphalan exposure in the vitreous, aqueous humor, and retina, as well as the pharmacokinetic parameter estimates, are reported in Table 1.

In all cases, melphalan concentrations in plasma were below the limit of quantitation of our analytical technique (10 ng/mL) at all times after intravitreal injection. This result supports the lack of systemic toxicity observed in the patients after intravitreal injections of melphalan.

Table 1. Melphalan Pharmacokinetic Parameter Estimates After Intravitreal Injection

Pharmacokinetic parameter/compartment	*Vitreous humor*	*Aqueous humor*	*Retina*
C_{max} (µg/mL or µg/g)	7.8	0.024	9.80
T_{max} (h)	[a]	0.80	0.25
AUC_{last} (µg * h/mL or µg * h/g)	14.03	0.0956	19.52
K_{el}/F (h^{-1})	0.71 (4.8)[b]	3.58 (63.5)	0.59 (7.4)
$T_{1/2}$ (h)	0.98 (4.8)	0.19 (63.5)	1.18 (7.4)
K_{ab}/F (h^{-1})	—	0.28 (39)	13.78 (50.7)

[a]Immediately after the injection.
[b]First-order elimination rate constant. Zero-order elimination rate constant was 2.61 (µg/mL)/h (9.8%).
C_{max}, model-predicted maximum concentration; T_{max}, time at maximum concentration; AUC_{last}, melphalan area under the concentration versus time profile up to the last observed data; K_{el}/F, apparent elimination rate constant; $T_{1/2}$, elimination half-life time; K_{ab}/F, apparent absorption rate constant; F, bioavailability factor ($F = 1$ for vitreous). Data are shown as mean estimates and in parenthesis, the coefficient of variation of the estimate (CV%).

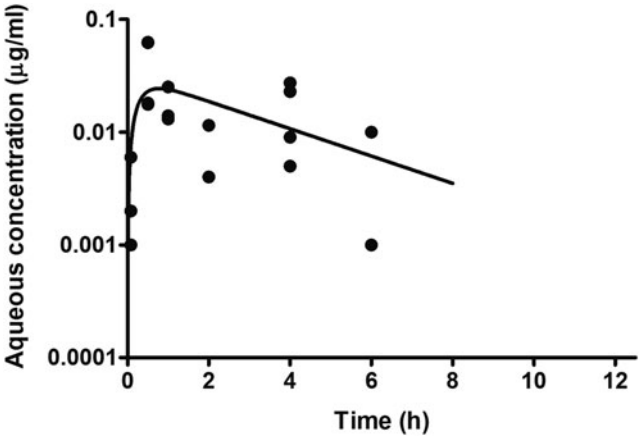

FIG. 2. Aqueous humor melphalan concentration over time after intravitreal injection of 15 µg. Data are shown as individual values and the line represents the model-predicted values.

Discussion

This study describes the disposition of melphalan after intravitreal injection to the rabbit eye. To our knowledge, no previous studies have evaluated these parameters, and dose calculation and schedules for administration in patients were based only on clinical and preclinical observations of activity and toxicity. Vitreous levels of melphalan exceeded the calculated pharmacological antitumor concentration for up to 1–5 h after the injection of 15 µg that correlates with the clinical dose when scaling to the vitreous volume, with low exposure of the aqueous humor and undetectable levels in plasma. In addition, melphalan attained maximum retina concentrations after only 0.25 h and levels were quantifiable up to 12 h after the injection.

At the end of the 1980s, the first evidence of sensitivity of retinoblastoma cell lines to melphalan exposure was reported.[15] After assessing melphalan toxicity in rabbits, the agent was introduced into the clinical practice delivered by intravitreal injections and selective ophthalmic artery infusion depending on whether seeds or retinal tumors were targeted.[7,16] Recently, intravitreal injection of chemotherapy has become a widespread procedure for the control of vitreous seeds in retinoblastoma treatment based on the development of a safe technique to prevent extraocular dissemination of tumor cells from the needle track.[13,14,21] Nonetheless, there are no previous reports on the characterization of the pharmacokinetics of melphalan after direct injection into the vitreous.

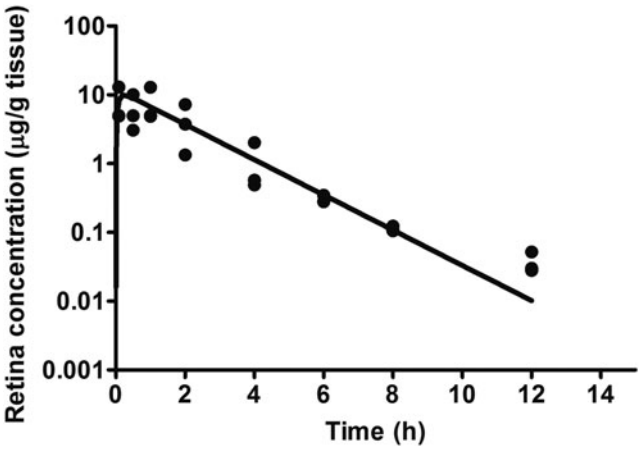

FIG. 3. Melphalan retina levels in rabbits. Individual levels of melphalan in the retina of the treated eyes are shown as *symbols* and the *line*, the model-predicted values.

After intravitreal injection, melphalan diffuses within the vitreous cavity and the observed volume of distribution in the rabbit eye ($V_{vit} = 1.9$ mL) was consistent with the physiologic volume of the vitreous of a rabbit.[18] The maximum vitreous concentration predicted by the pharmacokinetic model was 7.8 µg/mL. Interestingly, during the first 2 h, melphalan showed a zero-order elimination. In other words, the rate of elimination is independent of the drug concentration, which has been previously shown in cases when the elimination process is saturated.[22] In the specific case of intravitreal melphalan, this finding suggests that if the dose is increased, this may result in a longer time until the vitreous concentration declines enough to start a first-order elimination process and therefore potentially exposes the retina to toxic levels. It would be interesting to assess whether melphalan doses lower than 15 µg also lead to zero-order elimination from the vitreous. Another point to take into account is the spontaneous vitreous metabolism considering that melphalan undergoes hydrolysis to mono- and dihydroxymelphalan at physiological pH and body temperature.[23] In this sense, Bosanquet et al. reported that the half-life time of a solution of 20 µg/mL in a cell culture medium with 10% fetal bovine serum at physiological temperature was only 1.1 h.[23] In this study, the half-life immediately and 1 h after intravitreal injection was 1.5 and 1.0 h calculated according to a zero-order process. These values are close to the half-life reported *in vitro* for spontaneous hydrolysis of melphalan. Nonetheless, this was beyond the scope of this study, and the binding properties of melphalan to vitreous components as well as the concentration of the metabolites should be further assessed.

Two hours after intravitreal injection, melphalan concentrations declined according to a first-order elimination process with a half-life of 1 h. This result implies that every hour, the concentration of melphalan in the vitreous is reduced to one-half of its previous concentration. Therefore, no accumulation in this compartment may be expected if injected in a weekly fashion as used in clinical practice by many groups.[3,4,10,12,13] We observed that melphalan vitreous concentrations were above the concentration required to reduce *in vitro* cell viability by 50% (IC50, 1 µM, or 0.3 µg/mL in Y79 cell line) for up to 5 h after the injection, as the model-predicted vitreous concentration was about 0.3 µg/mL.[19] In addition, previously a report showed that a concentration of 4 µg/mL of melphalan could completely suppress colony formation by retinoblastoma cells *in vitro*.[7] Therefore, and based on our data, a complete cytotoxic effect in the vitreous could be expected for about 1 h after the intravitreal injection of a single dose of 30 µg. On the other hand, after superselective infusion of 7 mg of melphalan into the ophthalmic artery of the swine model, vitreous concentrations barely attained the IC50, and only at the end of the infusion, the maximum vitreous concentration was attained.[19] Altogether, these findings support the comparatively higher bioavailability of melphalan in the vitreous after intravitreal injection and the selection of this route for targeting vitreous seeds.

After distribution in the vitreous cavity, drugs may be removed by the anterior route through aqueous humor turnover.[18] Melphalan diffusion from the vitreous to the anterior chamber was fast with detectable levels in the aqueous humor 0.5 h after injection attaining a C_{max} of only 0.024 μg/mL at 0.80 h after injection. Moreover, we showed that melphalan was detectable between 0.5 and 6.0 h after the injection with low levels attained in this interval of time. Although it has been reported that for small molecules, such as melphalan, elimination occurs mainly through the anterior chamber, we observed that aqueous humor exposure was only 0.7% of that of the AUC attained in the vitreous. Similarly, our previous reports on topotecan intravitreal injection showed that drug exposure in the aqueous was only 5% of that in the vitreous compartment.[5] Thus, other routes or mechanisms of melphalan elimination from the vitreous compartment should be considered. The low exposure of the aqueous humor of rabbits to melphalan may be an important finding for translation into the clinical practice for patients with diffuse anterior chamber retinoblastoma. The presence of aqueous seeds in retinoblastoma eyes has traditionally been an indication for enucleation.[2] Nonetheless, a safe and conservative treatment alternative has recently been introduced into clinical practice with success to avoid enucleation of eyes with vision. The technique involves the intracameral injection of 15 μg of melphalan.[24] Our results support this procedure since if only an intravitreal injection for vitreous and aqueous seeds was performed, the low bioavailability of melphalan in the anterior chamber of the patient would be insufficient to achieve an antitumor effect. Therefore, it would be justified to treat both the anterior and posterior chamber independently according to tumor stage.

Finally, posterior elimination is the major route of drug elimination for small molecules through the retina and choroid capillaries.[18] Even though it was not calculated, retinal permeability should be low as melphalan attained the maximum concentration in this compartment only after 0.25 h. In addition, melphalan concentrations in the retina were still quantifiable 12 h after intravitreal injection even though no drug could be detected in the vitreous. Nevertheless, the presence of melphalan in the retina for 12 h may contribute to retinal toxicity.

Although elimination of melphalan from the vitreous through the choroid flow draining into the systemic circulation was expected, the rabbits were devoid of detectable plasma levels of the drug. The absence of detectable levels of melphalan in plasma is consistent with the lack of systemic adverse events previously reported in patients treated with intravitreal injections of this chemotherapeutic agent.[12,13,17] Furthermore, systemic exposure in children receiving this same dose or a dose escalated to the human vitreous volume would be even lower given the larger plasma volume of distribution compared with a rabbit. The present results are in line with those previously obtained for topotecan after intravitreal injection in rabbits showing very low systemic exposure.

To characterize the pharmacokinetics of melphalan in the vitreous and retina without disrupting the ocular structures and altering the pharmacokinetics, only 1 sample could be obtained per eye.[5] The rabbit has recently been described as an adequate and well-correlated model to study human pharmacokinetics of several intravitreally injected drugs.[18] Although we support the present statement, none of the reported drugs were studied in ocular tumors, and therefore, we acknowledge the limitation of working with a normal animal model for translating results to an eye affected by tumor. The tumor may disrupt the blood–retina barrier, and the amount of drug that can be taken up by the seeds could also lead to changes in the disposition of melphalan in the eye and systemic circulation.

In summary, after intravitreal injection of 15 μg of melphalan, vitreous concentrations decline with an elimination half-life of 1 h, while attaining pharmacologically active levels for at least 5 h post dosing. Retina concentrations were still detectable up to 12 h after the injection, but removal of melphalan from the aqueous humor is fast. Plasma exposure is undetectable and therefore few, if any, systemic adverse events may be expected in clinical practice. The present results support intravitreal injection to increase the bioavailability of the chemotherapeutic agent in the vitreous, weighing retina exposure to target potential tumors against toxicity and function impairment in the clinical setting.

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Author Disclosure Statement

No competing financial interests exist.

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