Pharmacokinetic Analysis of Melphalan after Superselective Ophthalmic Artery Infusion in Preclinical Models and Retinoblastoma Patients

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Purpose. To characterize melphalan pharmacokinetics after superselective ophthalmic artery infusion (SSOAI) in animals and children with retinoblastoma.

Methods. Vitreous and plasma samples of five Landrace pigs were obtained over a 4-hour period after SSOAI of melphalan (7 mg). Melphalan cytotoxicity was evaluated in retinoblastoma cell lines with and without topotecan. Plasma samples were obtained from 17 retinoblastoma patients after SSOAI of 3 to 6 mg of melphalan to one ($n = 14$) or two eyes ($n = 3$). Correlation between plasma pharmacokinetics and age, dosage, and systemic toxicity was studied in patients.

Results. In animals, melphalan peak vitreous levels were greater than its IC50 and resulted in 3-fold vitreous-to-plasma exposure. In patients, a large variability in pharmacokinetic parameters was observed and it was explained mainly by body weight ($P < 0.05$). A significantly higher systemic area under the curve was obtained in children receiving more than 0.48 mg/kg for bilateral tandem infusions ($P < 0.05$). These children had 50% probability of grades 3–4 neutropenia. Plasma concentrations after 2 and 4 hours of SSOAI were significantly higher in these children ($P < 0.05$). A synergistic cytotoxic effect of melphalan and topotecan was evident in cell lines.

Conclusions. Potentially active levels of melphalan after SSOAI were achieved in the vitreous of animals. Low systemic exposure was found in animals and children. Doses greater than 0.48 mg/kg, given for bilateral tandem infusions, were associated with significantly higher plasma levels and increased risk of neutropenia. Synergistic in vitro cytotoxicity between melphalan and topotecan favors combination treatment. (*Invest Ophthalmol Vis Sci.* 2012;53:4205–4212) DOI: 10.1167/iovs.12-9501

In recent years, superselective intraophthalmic artery infusion (SSOAI) of chemotherapeutic agents became an established therapy for advanced intraocular retinoblastoma, resulting in a dramatic increase of the preservation rate of these eyes.[1] The dose of melphalan administered by SSOAI is chosen empirically based on estimations of patient age, weight, vessel anatomy, and response to treatment.[2] However, despite its increased use in patients with retinoblastoma, there are few studies describing the pharmacokinetics of the drugs used. Melphalan was selected by Inomata and Kaneko[3] as the candidate drug for intraarterial administration based on its in vitro activity and became the most widely used agent for SSOAI. However, we are not aware of any pharmacokinetic study to characterize the agent delivered by this route. Despite strong clinical evidence supporting the efficacy of melphalan administered through SSOAI, little is known about basic facts, such as its ability to penetrate into the ocular structures including the vitreous, the optimal concentrations of the drug needed to exert an antitumor effect, or even the extent of systemic exposure after SSOAI. Studies in humans would be ideal to characterize these features, although fears of extraocular tumor dissemination make procurement of vitreous specimens difficult and thus only plasma pharmacokinetic studies are feasible. Current tumor-bearing animal models in mice and rabbits are not suitable for the study of ocular pharmacokinetics because their small size makes SSOAI technically difficult to perform. Thus, only large, non-tumor-bearing animals are available for pharmacokinetic studies of chemotherapeutic agents administered by SSOAI; thus, our group used the swine model for these studies.[4] Besides, a combination of melphalan with other chemotherapy agents, such as carboplatin or topotecan, has been used for increasing its cytotoxic effect in the clinical setting, and there are no experimental studies on drug combinations supporting their use.[1]

For a more complete understanding of melphalan pharmacokinetics we took a multimodal approach, combining data from cell lines, a swine model, and children with retinoblastoma including: (1) In vitro studies on mephalan alone and in combination with topotecan to determine inhibitory concentrations in retinoblastoma cell lines; and translating this to the potential antitumor activity of the drug levels attained in the eye. (2) Animal studies for a comprehensive pharmacokinetic

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characterization of melphalan in the vitreous, ocular tissues, and plasma after SSOAI. (3) Pharmacokinetic studies in children with retinoblastoma who have received SSOAI melphalan to characterize the systemic exposure of the drug and correlate the findings with hematopoietic toxicity and other parameters such as age, weight, and dose. (4) Pharmacokinetic models integrating all the described information for applications to the clinical setting.

Materials and Methods

Melphalan Cytotoxicity in Retinoblastoma Cell Lines

Retinoblastoma cell lines Y79 and WERI-RB were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with fetal bovine serum (FBS; Greiner Bio-one, Wemmel, Belgium), 2 mM L-glutamine, 1.5 gr/L $NaHCO_3$, 4.5 gr/L glucose, 10 mM HEPES, and 1 mM sodium pyruvate at 37°C in a humidified 95% air and 5% CO_2 atmosphere. Cells were counted with a hemocytometer, seeded in 24-well plates, and cultured for 24 hours. Each cell line was exposed to ten different concentrations ranging from 0.001 to 100.0 μM, while diluent was added to the control wells. Cells were incubated for 72 hours and viable cell number was determined for each concentration in triplicate. The viability of each cell line was assessed using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] colorimetric assay (Sigma-Aldrich, St. Louis, MO). Cell survival was determined based on the ratio of the absorbance in each well with respect to the control. Data from two experiments were combined to determine the proportion of viable cells at every drug concentration and the dose–response curve was calculated by means of nonlinear regression to obtain the maximum cell viability and the 50% inhibitory concentration (IC50) in both cell lines. Thereafter, we studied the effect of topotecan on the melphalan dose–response curve. Melphalan cytotoxicity was evaluated in both cell lines in the presence of topotecan at a fixed concentration corresponding to its IC50 that was previously determined by our group. To test for the pharmacologic interaction between the melphalan and topotecan combination in both cells lines, these data were analyzed by means of the method of Chou and Talalay using a commercial software program (CalcuSyn; Biosoft, Inc., Cambridge, UK) and the combination index was calculated. A combination index < 1 was considered a synergistic effect.[5]

Animal Studies

A total of five domestic Landrace pigs, weighing between 60 and 80 kg, were used in the present study complying with the tenets of Association for Research in Vision and Ophthalmology for the use of animals in ophthalmic and vision research. Animal sedation and SSOAI was performed as previously published.[4] Before chemotherapy infusion, a microdialysis probe was inserted into the vitreous of the chemotherapy-treated eye and another in the control eye using a 25-gauge needle through the conjunctiva.[4,6] PBS (pH 7.4) was used as perfusion fluid delivered at a flow rate of 1 μL/min using a microinfusion pump (KDS230; KD Scientific, Holliston, MA). After probe stabilization, 7 mg of commercial melphalan (Eriolan; Eriochem, Paraná, Argentina) were given into the ophthalmic artery by SSOAI in a pulsatile fashion over 30 minutes. Dialysates from vitreous humor were collected every 30 minutes over a period of 4 hours after melphalan administration. The recovery was determined by perfusing the probe with a concentrated melphalan solution (250 ng/mL) and estimating the recovery in vitro by the retrodialysis method. The mean recovery value obtained for the probes was 23 ± 9% (mean ±% coefficient of variation) and was used to estimate melphalan vitreous level based on each dialysate sample concentration. Simultaneously, the microcatheter used to administer melphalan was removed and arterial blood plasma samples were obtained at: 0.083, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 hours after the end of the infusion. Blood samples were immediately centrifuged and 100 μL of plasma was treated with 110 μL of cold methanol/HCL (10:1). After collecting the 4-hour vitreous dialysates and plasma samples, the animal was euthanized and both eyes were immediately enucleated. The retinal pigment epithelium (RPE)–choroid and retina tissues were dissected from both control and treated eyes and washed with cold PBS, weighed, and manually homogenized with a methanolic acid cold solution in a 1 to 6 dilution. Methanolic supernatant plasma extracts, dialysates, and tissues were stored at −20°C until assay.

Melphalan Analytical Assay

Melphalan concentrations were determined by HPLC coupled with a fluorescence detector set at excitation/emission wavelengths of 256 and 426 nm, respectively, after validating a modified method reported by others.[7] The linear ranges for plasma and vitreous assays were from 10 to 700 ng/mL and from 5 to 100 ng/mL, respectively. Interday precision was <11% for melphalan in methanolic extracts and vitreous except for the lowest concentration that had an interday precision < 16%.

Melphalan Clinical Studies

Children with relapsed or refractory intraocular retinoblastoma in whom SSOAI was given for salvage therapy after chemoreduction with systemic chemotherapy were included in the present study. The protocol followed the tenets of the Declaration of Helsinki and institutional review board approval was obtained. Children received 3 to 6 mg per eye of melphalan by SSOAI in a pulsatile fashion over 30 minutes for each eye. The initial dose was: 3 mg for children under 2 years of age, 4 mg for children from 2 to 3 years of age, and 5 mg for children older than 3 years of age.[1] Children in whom the drug was administered to both eyes (tandem therapy) received an initial calculated dose to each eye according to the same guideline up to a maximum of 0.5 mg/kg of total dose.[1,8] Based on toxicity and response, some children were given higher doses in subsequent cycles if clinically indicated. In children receiving tandem therapy, the catheter was retracted after infusing the first eye and then passed into the second ophthalmic artery for another 30-minute infusion. One milliliter of blood was collected in heparinized tubes from a peripheral catheter before starting melphalan administration and at the end of the infusion, 0.5, 1.0, 2.0, and 3.0 hours after finishing the infusion. For those patients treated with tandem therapy, blood samples were collected at the end of the infusion of each eye and at the mentioned times after finishing the administration. Once collected, blood samples were centrifuged and plasma was precipitated as previously described. Methanolic supernatants were stored at −20°C until melphalan analysis.

Patients were examined clinically 24 hours after the procedure and a complete blood cell count was done routinely at 10 and approximately 21 days after the procedure. After each chemotherapy cycle, hematologic toxicities were graded according to the Common Terminology Criteria for Adverse Events version 4.0.[9]

Pharmacokinetic Analysis

Melphalan plasma and vitreous concentration versus time data obtained in animals after SSOAI were simultaneously fitted under the assumption of a two-compartment model as previously described by our group using a commercially free software program (ADAPT software v.5; Biomedical Simulations Resource, Los Angeles, CA).[4] The pharmacokinetic parameters estimated included clearance (CL), volume of distribution of the central compartment (V_c) and the intercompartment rate constants (k_{cv}, k_{vc}), whereas the apparent volume of distribution of the vitreous compartment was fixed to 2 mL.[4] Maximum vitreous and plasma concentrations (C_{max}) were obtained from the observed data.

In human, the population pharmacokinetic analysis of melphalan was performed by means of nonlinear mixed-effects modeling method, implemented in commercially free software (Monolix v. 3.2, Berkeley, CA).[10] The pharmacokinetic parameters estimated included clearance (CL), volume of distribution of the central and peripheral compartment (V_c and V_p, respectively), and intercompartmental clearance (Q) assuming a log-normal distribution. The residual error was evaluated using a mixed proportional and additive error model. Patient-specific characteristics (covariate) such as age, body weight, and body surface area were evaluated for their significance in the model to explain to some extent the interindividual variability observed in the parameters. A covariate was considered significant if its addition to the base model reduced the objective function value by at least 3.84 units ($P < 0.05$) and the coefficient that relates the covariate and the pharmacokinetic parameter was significantly different from zero ($P < 0.05$). In addition, we evaluated graphically and by means of linear mixed-effects modeling, the relationships between the individual estimates of the pharmacokinetic parameters and the covariates. The area under the plasma concentration versus time profile (AUC) for each cycle that melphalan pharmacokinetics was evaluated was obtained after simulating the concentration–time data based on the estimated individual pharmacokinetic parameters and then calculated using the log-linear trapezoidal rule. Finally, the AUC, C_{max}, and melphalan plasma concentration after 2 and 4 hours of starting the infusion were compared between unilateral and tandem administration by means of repeated-measures ANOVA at a significance level of $P < 0.05$.

Results

Melphalan Cytotoxicity Studies

In vitro sensitivity to melphalan calculated as the 50% inhibitory concentration (IC50), was 1.07 and 1.63 μM in Y79 and WERI-RB cells, respectively (Fig. 1). As shown in Figure 1 (insets), at a topotecan concentration corresponding to the IC50 (10 nM; data not shown), the combination of melphalan and topotecan did not modify melphalan IC50 ($P > 0.1$). Melphalan IC50 alone and in the combination was 1.07 and 0.95 μM in Y79 and 1.63 and 0.96 μM in WERI-RB, respectively. The maximum cell viability (SE) was reduced in Y79 and WERI-RB from 111.1% (2.2) and 90.3% (4.9) to 60.4% (2.3) and 48.9% (1.0), respectively ($P < 0.001$). We observed a synergistic effect of the combination of topotecan and melphalan in both cell lines when exposed to melphalan at 1 μM and higher concentrations and topotecan at its IC50 (combination index < 1).

Ocular and Systemic Pharmacokinetics of Melphalan in Animals

Preclinical melphalan pharmacokinetics was characterized in five Landrace pigs after SSOAI. One animal died 30 minutes after the end of the drug infusion and, thus, the obtained data from that animal were not considered for statistical analysis. In all cases, melphalan vitreous C_{max} was observed in the first interval of collection samples corresponding to 0 to 30 minutes after SSOAI. In addition, C_{max} in plasma was obtained at the end of melphalan infusion. The obtained C_{max} values in vitreous and plasma were [median (range)] 170.1 ng/mL (47.3–416.2) and 56.9 ng/mL (39.2–114.8), respectively. As shown in Figure 2, melphalan vitreous concentrations after the end of the SSOAI were higher than plasma levels during the first 3 hours for all but one animal. However, plasma and vitreous concentrations were almost the same 4 hours after the end of infusion, being approximately 15 ng/mL. The derived median (range) melphalan pharmacokinetic parameter estimates for the studied animals were: CL: 18.2 L/h (7.6–29.6); V_c: 56.5 L (29.0–71.6); k_{cv}: 0.0035 h^{-1} (0.0001–0.0043); k_{vc}: 28.9 h^{-1} (2.1–41.9).

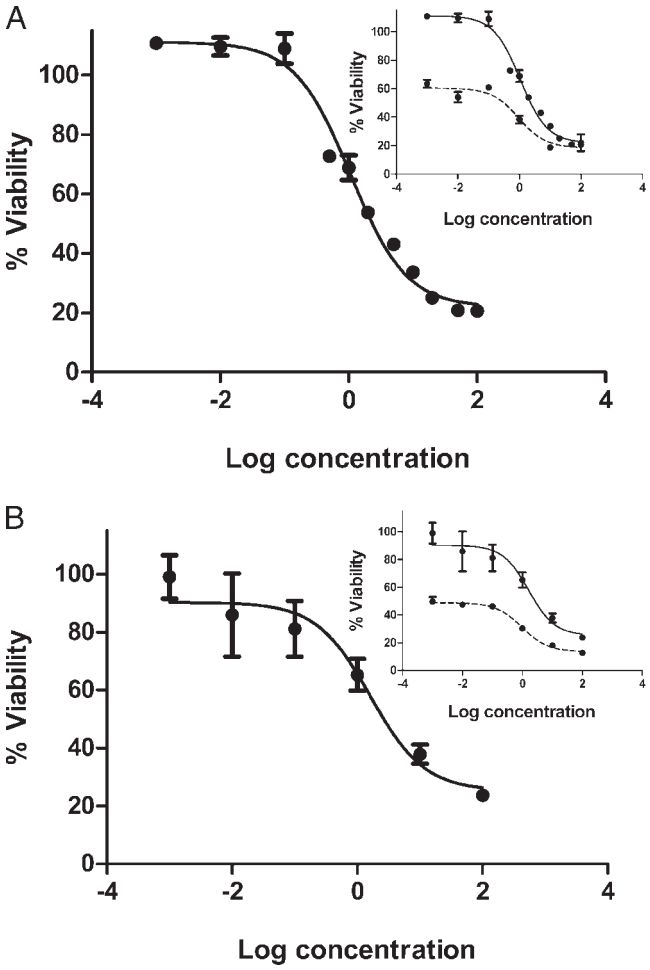

Figure 1. Dose-dependent cytotoxicty of melphalan in (**A**) Y79 and (**B**) WERI-RB retinoblastoma cell lines. Data are presented as mean ± SEM of duplicate experiments done in triplicates. The *insets* show the effect of topotecan 10 nM on melphalan cytotoxicty of both cell lines.

The calculated median (range) melphalan AUC in vitreous and plasma was 391 ng·h/mL (163.4–472.3) and 134.1 ng·h/mL (101.9–284.5), respectively. Thus, the vitreous exposure to melphalan was approximately 3-fold greater than that obtained in plasma, with the ratio between median (range) vitreous to plasma exposure being 3.1 (range 0.6–4.2). As previously described for the AUC, the median (range) ratio between vitreous to plasma C_{max} as a measure of penetration into the vitreous was 3.4 (0.4–7.9). Melphalan was detected in the RPE-choroid in three of four animals with a median (range) of 295.2 ng/g tissue (207.9–528.9). However, it was detected in the retina of only one animal at a concentration of 516.2 ng/g tissue. No melphalan could be detected in the retina or RPE-choroid of the contralateral eye.

Melphalan Pharmacokinetics in Children with Retinoblastoma

A total of 36 cycles of chemotherapy from 17 patients were evaluated in the present study. Patient characteristics and demographics are summarized in Table 1. For developing the pharmacokinetic model, we used a total of 131 melphalan plasma concentrations. A two-compartment model with first-

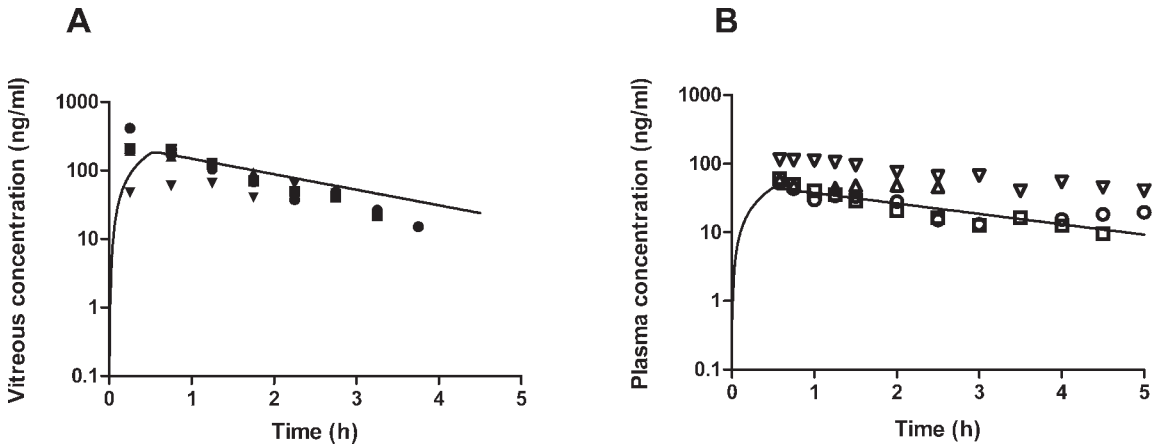

Figure 2. Concentration-versus-time profiles in (**A**) vitreous humor of treated eyes and (**B**) plasma of pigs after ophthalmic artery infusion of 7 mg of melphalan. *Full* and *empty symbols* represent individual data points for vitreous and plasma concentrations, respectively, and the *lines*, the best-predicted concentrations for a representative animal.

order elimination was used and adequately fitted the data (see Fig. 3A for a representative patient receiving the drug to one eye only). In children receiving tandem therapy, we noted that the interval of time marking the end of infusion of the first eye to the beginning of the infusion into the second eye had an impact on the drug disposition in plasma as shown in Figure 3B. In delayed tandem therapy with that long interval, a bimodal profile would be observed. This was in contrast to a classical constant infusion one-peak profile found in short intereye times where elimination from the body is limited. As melphalan was administered to the same patient in multiple occasions, interoccasion variability was also incorporated in the base model. The population pharmacokinetic parameters obtained for the final model are presented in Table 2. A large interindividual variability was observed accounting for 56% and 52% for CL and V_c, respectively. The covariate analysis showed that interindividual variability in CL and V_c could be explained in part by age, weight, and body surface area ($P < 0.05$). For the final model, only weight was considered because it explained 81% and 75% of the IIV in CL and V_c, respectively. A relationship between systemic exposure to melphalan (AUC) and dose corrected by weight was observed as depicted in Figure 4. Interestingly, as shown in Figure 5A, we observed a significantly higher AUC in children receiving more than 0.48 mg/kg of melphalan ($P < 0.05$). In addition, melphalan plasma concentrations after 2 and 4 hours of starting drug infusion significantly differed between children receiving SSOAI to one eye compared with those receiving tandem therapy, as represented in Figures 5B, 5C ($P < 0.05$).

Table 1. Patient Characteristics and Demographics at the Start of Therapy

Characteristics	Median (Range)
Age (y)	1.8 (0.6–6.2)
Weight (kg)	11.4 (8.8–20.5)
BSA (m^2)	0.5 (0.4–0.8)
	Number of patients ($n = 17$)
Sex	Male: 5 Female: 12
	Number of patients with previous treatments
Systemic chemotherapy	17 (median 5 cycles of carboplatin, etoposide, and vincristine and 1 case received 5 cycles of topotecan as second line therapy)
SSOAI*	5
	Number of cycles
Type of administration	Unilateral: 30 Tandem: 6
Dose† (mg)	3 mg: 7 4 mg: 6 5 mg: 14 6 mg: 6 7 mg: 3

* SSOAI, superselective ophthalmic artery infusion with a different chemotherapeutic agent (topotecan/carboplatin) than melphalan administered in previous cycles to the present pharmacokinetic evaluation.

† The presented data are the total doses administered in unilateral and bilateral injections.

Simulated Data on the Impact of Dose Reduction in the Vitreous Exposure

Based on our findings about increased risk of hematopoietic toxicity in children receiving tandem therapy, we estimated the impact of dose reduction on melphalan concentrations in the vitreous using a hypothetical case of a 1- to 3-year-old patient weighing 10 kg with bilateral retinoblastoma. Calculations were made using the mean population pharmacokinetic parameters from patients and the intercompartment rate constants between the plasma and vitreous compartments obtained from the animal studies. An exposure of AUC_{pl}: 1507.8 ng/mL and AUC_{vit}: 105.4 ng/mL would be obtained if a dose of 4 mg per eye were administered, based on dose assignment by age. If reducing the dose to our proposed limit of 0.48 mg/kg (2.4 mg per eye in the present hypothetical case) to avoid hematopoietic toxicity, the exposure would decrease by approximately 40% (AUC_{pl}: 904.7 ng/mL and AUC_{vit}: 63.3 ng/mL).

Toxicity

SSOAI of melphalan was well tolerated and very few adverse events were recorded. Grade 3 neutropenia was observed in four cycles in four patients and grade 4 neutropenia was

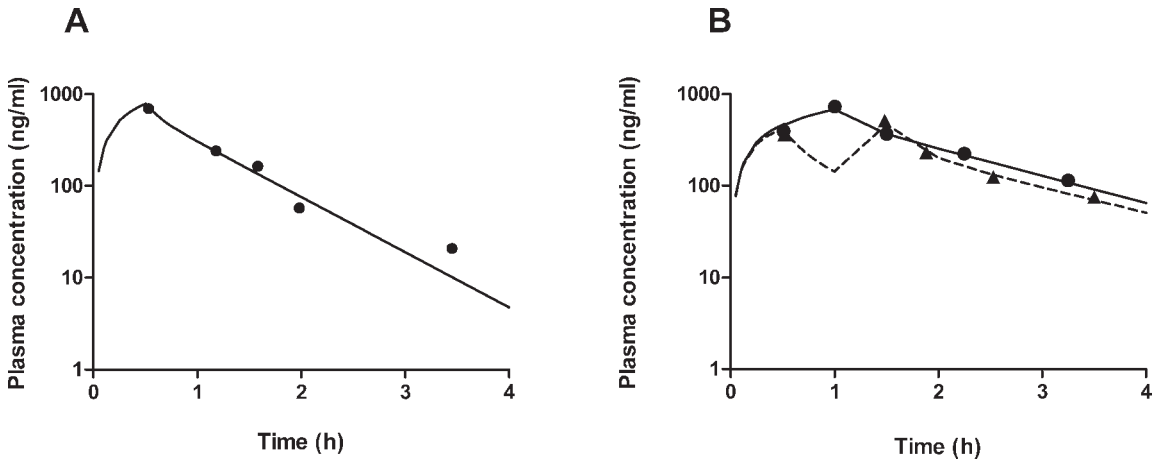

Figure 3. (**A**) Concentration versus time profile after superselective ophthalmic artery infusion of 5 mg in a unilateral retinoblastoma patient and in (**B**) two tandem administrated patients with short (*solid line*) and long (*dashed line*) interval of time between the end of the first eye and the start of the second eye infusion. In the latter case, the interval of time between the two eyes infusion was 30 minutes. In all cases, the *symbols* represent melphalan plasma observed concentrations and the *lines*, the model-predicted concentrations.

observed in two cases. Three of the four patients who received tandem therapy had neutropenia. Specifically, in 50% of the cycles where patients received tandem therapy with dosages > 0.48 mg/kg, we observed grade 3 or grade 4 neutropenia. Transfusions were not required in any case. The ophthalmologic toxicity was mild and included orbital edema in three cycles and madarosis in two patients.

Discussion

Our study shows that potentially active levels of melphalan were achieved in the vitreous of a non-tumor-bearing animal after SSOAI. Data from our patients with retinoblastoma showed a plasma concentration versus time profile that was comparable to our animal model when corrected by weight, confirming a low systemic exposure to the drug in children receiving this treatment. Although children with retinoblastoma have been treated with melphalan by SSOAI, the present report is the first pharmacokinetic study of melphalan using this superselective route of drug administration in this population.

The pharmacokinetic features of melphalan after intravenous infusion[11,12] and local administration, such as isolated limb perfusion for the treatment of melanoma,[13] have been well characterized. However, to our knowledge, there are no data regarding its pharmacokinetics after SSOAI in patients or animals. It is evident from clinical practice, where retinoblastoma tumors respond dramatically to melphalan SSOAI monotherapy, that the drug is able to reach active levels in the eye. However, whereas eyes with retinal detachment and subretinal seeds respond particularly well to SSOAI melphalan,[14,15] progressive tumors in previously irradiated eyes and those with vitreous seeding show a less impressive response and remain difficult to cure.[15] Therefore, it would be useful to gain knowledge on the vitreous levels of melphalan to help guide treatment, particularly in cases where aggressive therapy may be necessary.

According to our in vitro sensitivity studies in retinoblastoma cell lines and other previously reported data,[16] melphalan concentrations of 1–1.6 μM are needed to exceed its IC50. These levels were attainable in the vitreous of our swine model following 7 mg of melphalan via SSOAI, but only at the C_{max} and declined shortly thereafter. This relatively low efficiency of melphalan to reach the vitreous might be related to its poor affinity to the L-type amino acid transporter 1 (LAT1), which mediates its transport through the blood–retinal barrier.[17]

In a previous study, we found that topotecan permeated into the vitreous cavity of the same animal model with greater efficiency than melphalan by 5- to 10-fold since its relative

Table 2. Melphalan Population Pharmacokinetic Parameters after SSOAI in Retinoblastoma Patients

Parameter	Final Model (Mean, SE)
CL (L/h/kg)	0.51 (0.03)
V_c (L/kg)	0.18 (0.04)
Q (L/h/kg)	0.93 (0.15)
V_p (L/kg)	0.25 (0.03)
AUC/D (ng·h/mL)/mg*	165.5 (83.8–397.6)
C_{max}/D (ng/mL)/mg*	181.1 (69.7–340.7)

* Data are shown as median (range). For C_{max}, data are shown for unilateral administrations. For bilateral administrations, C_{max}/D (median, range) was: 91.5 ng/mL/mg (57.9–182.4). CL, clearance; Q, intercompartmental clearance; V_c, V_p, volume of distribution of the central and peripheral compartment, respectively; AUC/D: area under the concentration versus time profile corrected by dose (D); C_{max}, maximum plasma concentration corrected by dose (D).

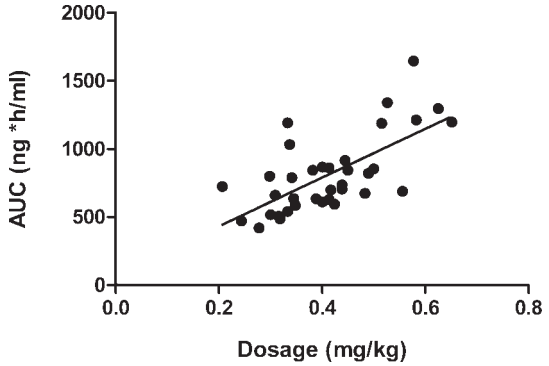

Figure 4. Relationship between systemic exposure and dosage (dose expressed per kg of total body weight). The *symbols* represent individual melphalan systemic exposure and the *line*, the model-predicted concentrations considering a linear mixed-effects approximation.

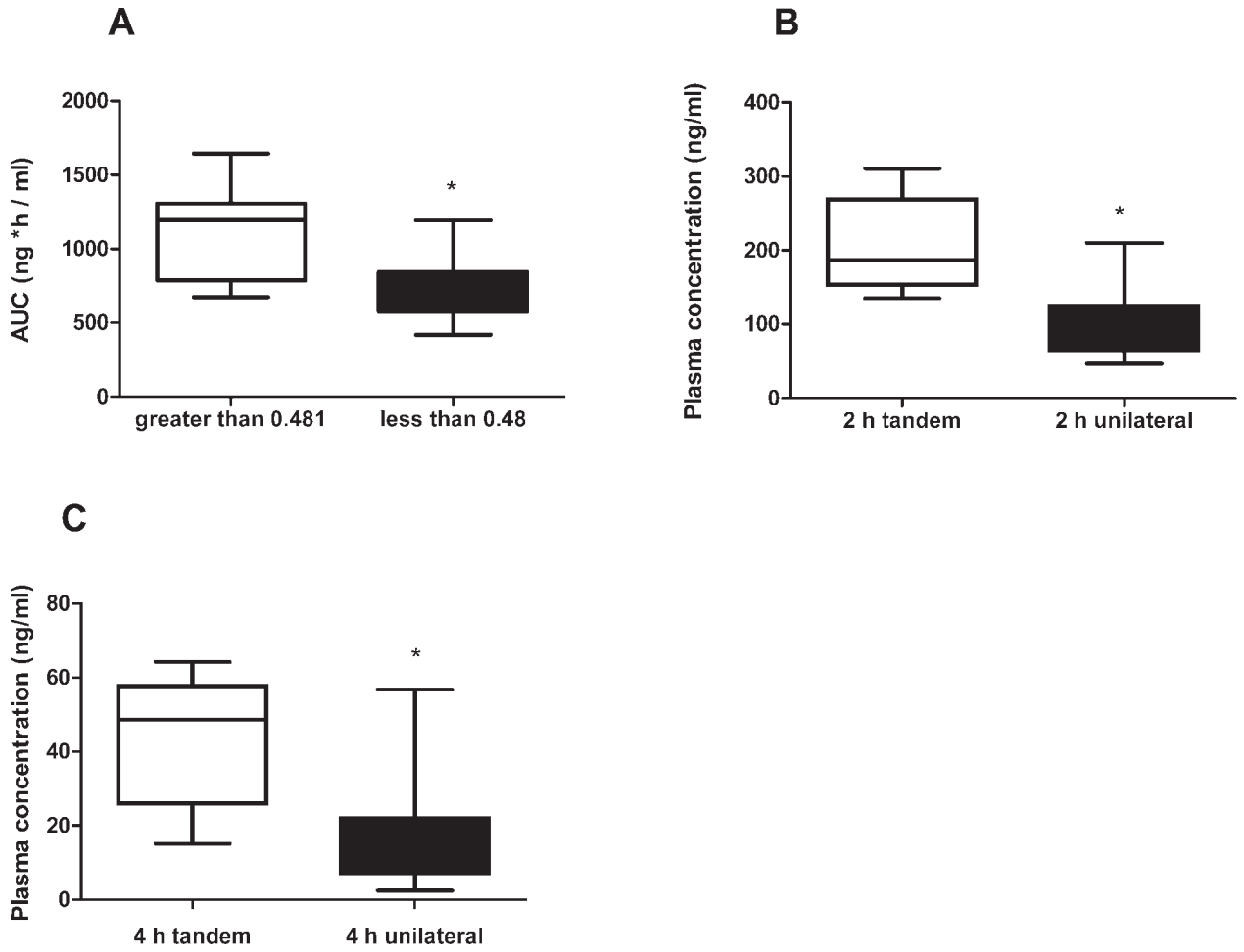

Figure 5. Boxplot of (**A**) systemic exposure of melphalan (AUC) and dosage; (**B**) plasma concentration after 2 hours; and (**C**) after 4 hours of SSOAI in (*empty*) tandem and (*bold*) unilateral therapy. The *horizontal line* corresponds to the median; the *box*, the quartiles; and *whisker*, range of the data. $^*P < 0.05$.

median vitreous-to-plasma AUC (AUC_{vit}/AUC_{pl}) and C_{max} ($C_{max,vit}/C_{max,pl}$) after SSOAI was 29 and 15.4, respectively.[4] On the contrary, in the present study we showed that after melphalan SSOAI, the relative median AUC_{vit}/AUC_{pl} and $C_{max,vit}/C_{max,pl}$ were 3.2 and 3.4, respectively. Besides, topotecan concentrations in the vitreous at 4 hours following SSOAI were still well above its IC50,[4] as opposed to melphalan when given as single agents to pigs. Whether this higher vitreous with respect to plasma exposure represents a favorable penetration of topotecan through the blood–retinal barrier into the vitreous, or a lower clearance of the drug from the vitreous back into the general blood circulation could not be ascertained by our data. Therefore, the passage of melphalan through the blood–retinal barrier to the vitreous is relatively inefficient compared with other drugs like topotecan, another candidate drug for SSOAI.

There are at least two limitations from our study that need to be considered. Our model was a non-tumor-bearing animal with intact retina and tumor-induced disruption of the blood–retinal barrier may increase the drug penetration to the vitreous in the clinical scenario.[18] In addition, IC50 was estimated from commercial retinoblastoma cell lines that may have a different chemosensitivity to the tumor cells in vivo.[3,19]

Our in vitro sensitivity studies showed that topotecan and melphalan are synergistic against retinoblastoma cell lines as seen with the association of an alkylating agent and a topoisomerase inhibitor in other pediatric neuroectodermal malignancies.[20] Thus, although the vitreous penetration of melphalan may not be optimal, its combination with topotecan could be an attractive option to optimize the pharmacologic treatment when the vitreous is the main target.

In addition to the vitreous, we also estimated melphalan concentration in other eye structures at the time of enucleation. Interestingly in three of four animals, melphalan was still detectable in the RPE–choroid in the infused eye at 4 hours, but was absent from the retina. As seen in melanoma cells, melphalan (L-phenylalanine mustard) may be taken up preferentially by pigmented tissues such as the RPE since phenylanine is a metabolite of melanin.[21] However, its efflux from the RPE to the retina is inefficient due to its poor affinity to its specific LAT1 transport protein.[17] Our data suggest that melphalan accumulates in the RPE and perhaps this might play a role in the choroidal toxicity that has been reported for this drug.[22,23]

The present study also provided data about the systemic exposure to melphalan after SSIAO in children with retinoblastoma, suggesting that melphalan shows linear pharmacokinetics in the range of dosages that were evaluated. The estimated population pharmacokinetic parameters are in close relationship with those previously obtained after intravenous administration in children with malignant disease undergoing stem cell transplantation,[12] where a clearance and volume of distribution of 10.1 L/h and 4.8 L respectively were obtained in their population with a median weight of 18.8 kg.[12] Thus, by

correcting for weight as we propose, the pharmacokinetic parameters obtained after almost a 10-fold higher intravenous dose would be comparable to those obtained in the present study (Table 2). As was described for other local routes of administration of melphalan, such as regional isolated limb perfusion,[24] systemic exposure following SSOAI was low. This corroborates with the clinical experience where only occasional mild myelotoxicity is found at the dosages used. With a fixed dose, a wide interindividual variability in the clearance of melphalan translates in a wide variability of systemic exposure. Thus, identifying the sources of interpatient variability may provide better estimates of systemic toxicity. We found that there was a significant correlation between melphalan systemic exposure (AUC) and the dose corrected by corporal weight (Fig. 5). In the literature, doses ranging from 3 to 7.5 mg were proposed, but there is no general agreement on the dose among different groups.[25,26] Many factors, including vascular anatomy, wedge flow, and clinical response to treatment all influence the dose of chemotherapy to be chosen for SSOAI. Dose-related myelotoxicity is the most frequent side effect of intravenous melphalan.[27,28] However, in all reports of SSOAI of melphalan, only mild neutropenia is reported.[26] Gobin and colleagues[1] suggest a limit of 0.5 mg/kg of melphalan for SSOAI, and accordingly we found that children receiving a dosage > 0.48 mg/kg had a significantly higher AUC and a 50% chance of presenting grade 3 to grade 4 neutropenia compared with 0% of the cycles in children with lower dosages.

Therefore, a dose corrected by weight may be useful for clinical practice, particularly when tandem therapy or dose increments are considered. In those cases, clinicians prescribing dosages > 0.48 mg/kg should be aware of the potentially severe neutropenia. Even though limiting the dose would be associated with lower risk of hematopoietic toxicity, it may consequently reduce the amount of drug delivered to the eye. To estimate this, we compared the systemic and vitreous exposure of a hypothetical patient receiving a reduced dose to both eyes with a case receiving the full dose recommended by age and weight.[1] Although dose reduction to 0.48 mg/kg would theoretically be associated with a low risk of hematopoietic toxicity, it would also be accompanied with a 40% reduction in the vitreous exposure compared with the full dose. Therefore, combination therapy with topotecan may serve as an attractive option to decrease hematologic toxicity and potentially enhance the pharmacologic effect. Finally, we showed that higher melphalan plasma levels at 2 and 4 hours after the end of SSOAI were significantly associated with increased risk of myelotoxicity. Whether these concentrations could be used as predictors of hematologic toxicity after SSOAI should be defined in further studies.

Our data show that there is a low systemic exposure to melphalan after SSOAI, suggesting a low risk for associated sterility and induction of secondary leukemias. Although little is known about the threshold for gonadal toxicity in young children receiving melphalan or other alkylating agents,[29] data from patients receiving melphalan for stem cell transplantation show that dosages of 160 mg/m^2 (approximately 5.3 mg/kg) can cause sterility.[12] However, this exposure may be achieved after only 10 to 12 SSAOIs, particularly if higher doses of melphalan are used. Cases of therapy-related leukemia have been reported in children receiving systemic chemotherapy for retinoblastoma, including alkylating agents.[30] Secondary leukemia was also reported in adults receiving melphalan for multiple myeloma[31] and children receiving melphalan for the treatment of solid tumors.[32] In these cases, higher cumulative doses were typically used, leading to higher systemic exposure compared with children receiving melphalan given by SSOAI.

In conclusion, our data may help in determining the optimal dose of melphalan administered by SSOAI. Vitreous levels in a non-tumor-bearing swine model were close to the IC50 determined in two retinoblastoma cell lines. Despite that, melphalan penetrated the vitreous with relatively lower efficiency compared with other drugs like topotecan. SSOAI melphalan dosages > 0.48 mg/kg were associated with statistically significant increased systemic exposure to the drug, leading to a significantly higher risk of neutropenia in children with retinoblastoma. Based on the present findings and our in vitro data suggesting a synergistic effect, a drug combination of melphalan and topotecan might be a preferable alternative to escalating the dosage of melphalan for SSOAI in present retinoblastoma treatments.

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