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Method Article

Complete preclinical platform for intravitreal chemotherapy drug discovery for retinoblastoma: Assessment of pharmacokinetics, toxicity and efficacy using a rabbit model^{*}



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ABSTRACT

Current melphalan-based intravitreal chemotherapy regimens for retinoblastoma vitreous seeds are effective, but cause significant ocular toxicity. We describe protocols for each step of a drug discovery pipeline for preclinical development of novel drugs to maximize efficacy and minimize toxicity. These protocols include: 1) determination of vitreous pharmacokinetics *in vivo*, 2) *in vitro* assessment of drug cytotoxicity against retinoblastoma based on empiric pharmacokinetics, 3) back-calculation of minimum injection dose to achieve therapeutic concentrations, 4) *in vivo* determination of maximum-tolerable intravitreal dose, using a multimodal, structural and functional toxicity-assessment platform, and 5) *in vivo* determination of drug efficacy using a rabbit orthotopic xenograft model of retinoblastoma vitreous seeds. We likewise describe our methodology for direct quantitation of vitreous seeds, and the statistical methodology for assessment of toxicity and efficacy in evaluating novel drugs, as well as for comparisons between drugs.

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- Multi-step pipeline for intravitreal chemotherapy drug discovery for retinoblastoma, using novel rabbit models.
- Detailed protocols for determination of vitreous pharmacokinetics, calculation of optimal dose to inject to achieve therapeutic vitreous levels, determination of maximum tolerable dose using a novel complete toxicity-assessment platform, and *in vivo* efficacy against retinoblastoma using methodology to directly quantify vitreous tumor burden.
- Associated statistical methodology is also presented.

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Name and reference of original	N/A (see Reference section)
method	
Resource availability	N/A

Background

The most difficult-to-treat aspect of intraocular retinoblastoma are the vitreous seeds [1-4] – pieces of the tumor that break off and float in the vitreous jelly of the eye – which are not vascularized, thereby reducing systemic drug penetration to these parts of the tumor. The recent introduction of direct intravitreal injection of chemotherapy [5–8], to bathe the cells directly in chemotherapy, has revolutionized the treatment of retinoblastoma, and significantly increases the rate of tumor control and globe salvage in these advanced eyes with vitreous seeds [9–14]. However, the main chemotherapy agent used for intravitreal injections for retinoblastoma is melphalan. Since melphalan is highly toxic to the retina [9,15], intravitreal injections of melphalan are associated with visual functional loss [9,16–20].

Overview of approach

In order to develop newer, less toxic and still highly-effective alternative chemotherapies for intravitreal injection to eradicate retinoblastoma vitreous seeds, we have developed a preclinical drug discovery platform and step-by-step pipeline. In the present paper, we describe the methodology for the various components of this pipeline, including the determination of intravitreal pharmacokinetics for a given drug, calculation of the starting injection dose, determination of the maximum tolerable dose *in vivo* in a rabbit model using a complete toxicity-assessment platform, and confirmation of *in vivo* efficacy against retinoblastoma xenografts in the rabbit model.

Our overall approach is as follows:

- 1) Determine vitreous pharmacokinetics of intravitreally-injected drug-of-interest *in vivo*, including vitreous half-life.
- 2) Determine cytotoxicity profile for human retinoblastoma cells *in vitro*, based on a transient drug exposure equal to 5 vitreous half-lives, and calculate the IC_{90} .
- 3) Back-calculate the initial injection dose that would be required to maintain the IC_{90} concentration for 5 full half-lives on the far side of the vitreous from the injection point, based on the empiric pharmacokinetic parameters from Step 1 (referred to as the "IC₉₀ dose").

- 4) Starting from this IC90 dose, determine the maximum tolerable dose (MTD) of chemotherapy that does not cause retinal or retinal microvascular functional or structural damage. We use a toxicity assessment platform [15] that incorporate a plethora of various retinal toxicity testing strategies, including clinical examination, electroretinography, fundus photography, intravenous fluorescein angiography, optical coherence tomography, and optical coherence tomography angiography, along with histopathology.
- 5) Once the MTD has been determined, we perform *in vivo* efficacy experiments, whereby human vitreous seed xenografts are created orthotopically bilaterally in the eyes of rabbits [21], and then treated with a series of three weekly intravitreal injections of the MTD dose of the drugof-interest (*vs.* saline in the contralateral control eye). Following treatment, residual vitreous seed xenograft cells are harvested and quantified.

Each of these steps is described in detail below, along with statistical methodology and considerations employed for each.

Methods

Statement of research ethics

All animal experiments were performed under the auspices of the Vanderbilt Institutional Animal Care and Use Committee and adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement on Animal Use and the EU Directive 2010/63/EU for animal experiments.

Determination of intravitreal pharmacokinetics

- 3-6 New Zealand white rabbits (2.8-3.0 kg) are used.
- Insert 20-gauge valved vitrectomy cannula 2–3 mm posterior to the limbus superiorly. Preplacement of a valved cannula maintains the stability of the eye and prevents efflux of vitreous contents during manipulations [21].
- Inject 1 μ g of drug into the vitreous cavity inferiorly, 2–3 mm posterior to the limbus.
- Place rolled up towel under the rabbit's shoulders/neck so that the head rolls back (so injection site becomes superior to sampling site). Normally, in humans, injections are performed superiorly, and human patients are upright, so distribution across the vitreous cavity is (theoretically) aided by gravity. However, in the rabbit, it is significantly easier to perform vitreous taps superiorly in the eye, and the rabbit remains asleep and recumbent throughout the entire vitreous sampling experiment. To recapitulate the gravity-dependent situation that occurs in patients, the rabbit's head is allowed to roll back slightly by placing a rolled towel under the shoulders.
- Samples (~0.1 mL each) are obtained using a 21-gauge needle advanced through the preplaced cannula. The rabbit is temporarily taken off the rolled towel for these sampling procedures. Do not reach across the vitreous to intentionally sample from the side of the injection. Rather, sample from the opposite side relative to the injection site (*i.e.*, closer to the side of the cannula).
- Samples are obtained at 15 min, 30 min, 1 hour, 2 h, 4 h, and 6 h post-injection, and immediately placed on dry ice before transferring to -80 °C freezer for storage.
- The methodology for determination of drug concentration in vitreous samples has been described previously [21]. Briefly, vitreous samples are thawed and spiked with an internal carbamazepine standard, diluted with blank plasma, and deproteinized with acetonitrile. Calibration samples are prepared in parallel by spiking blank plasma with internal standard and known concentrations of drug. Samples are analyzed on a Thermo Scientific TSQ Quantum Ultra mass spectrometer interfaced to a Waters Acquity UPLC system.
- Drug concentrations are averaged across rabbits at each time point, and the resulting mean time-concentration data from each matrix are analyzed via non-compartmental analysis (Phoenix® WinNonlin® v6.4, Pharsight/Certara USA Inc., Princeton, NJ) to determine pharmacokinetic (PK) parameters, including half-life.

In vitro determination of dosing

- Human WERI-Rb1 retinoblastoma cells (ATCC, Manassas, VA) were grown in RPMI with 10% FBS and 1% sodium pyruvate under normal growth conditions (37 $\,^{\circ}$ C and 5% CO₂).
- 5000 cells in a total volume of 100 μ L were plated in each well of a 96-well black plate with a clear, flat bottom and allowed to grow for 3 days. Five replicates were plated for each concentration of drug.
- Study drug was added at various concentrations for a length of time equivalent to 5-times the empiric half-life as determined by the above-described pharmacokinetic experiments.
- After this time, cells were placed in a microcentrifuge tube and spun down. Media was aspirated, and the resulting cell pellet was resuspended in 100 µL normal growth media.
- After 7 days, live cells were counted using the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI). To each well, 20 μ L of CellTiter Blue reagent was added, and the plate was incubated for 4 h in the cell culture incubator. Stop solution was added to each well. The plate was read with an excitation wavelength of 560 nm and an emission wavelength of 590 nm in a SpectraMax plate reader.
- Survival curves were graphed with GraphPad, and IC₅₀ and IC₉₀ were calculated.

Calculation of intravitreal injection dose

To calculate the initial dose of drug that would have had to have been injected originally to achieve the IC_{90} (at the end of 5 half-lives at the farthest point in the vitreous relative to the injection site), we used the below approach:

From *in vivo* pharmacokinetic experiments, we know the half-life and the peak concentration actually achieved. For the below example, we will use actual data from a previous experiment using intravitreal belinostat.

- PK experiments (1 μ g into the vitreous) showed a half-life of 0.8 h
- There was near-immediate biodistribution across the vitreous, with the peak vitreous concentration of 0.457 mcg/mL occurring at 30 min.
- The theoretical C_{max} (assuming injection of 1 mcg into the 1.4 mL vitreous) would be 0.7143 mcg/mL. Thus, only 64% reaches the opposite side during the diffusion across the eye.
- We found the IC_{90} to be equal to ~8 micromolar, when WERI cells were exposed to 5 half-lives (4 h) of belinostat (and counted at 7 days post treatment).
- MW of belinostat is 318 g/mole

THUS:

- 8 micromolar equals 2546.8 mcg/L
- But the rabbit vitreous volume is only 1.4 mL (so divide by 714, which is 1000/1.4 mL)
- So this would be 3.567 mcg injected into the vitreous volume of 1.4 mL.
- \bullet This assumes it all makes it across the vitreous, but we know that only 64% of the C_{max} is actually achieved at the opposite side.
- 3.567 mcg divided by 0.64 = 5.57 mcg
- This is needed at the end of 5 half-lives, so multiply by 32 $(2 \times 2 \times 2 \times 2 \times 2)$
- Therefore, we calculate that 178 mcg initial injection dose maintains the IC90 at the far side of the vitreous for a full 5 half-lives.

Assessment of ocular toxicity and determination of maximum tolerable dose in vivo in rabbits

General considerations

Select appropriate doses. For example, based on the above calculations, one will have determined the injection dose that achieves the IC_{90} concentration at the far side of the vitreous for a full 5 half-lives. Begin with this dose (the "IC₉₀ dose"). If no toxicity is found, one can proceed to a higher dose, for example $2xIC_{90}$ dose, $4xIC_{90}$ dose, etc., to determine the maximum tolerable dose.

Various methodologies for stepwise progression to determine the maximum tolerable dose have been published. If the IC_{90} dose causes unacceptable toxicity, reduce the dose. A saline control group should be included.

Specific methodology

Retinal structure and function were assessed with our previously-described toxicity assessment platform [9,15]. Briefly, testing consisted of electroretinography (ERG), clinical ophthalmic examination, fundus photography, intravenous fluorescein angiography, optical coherence tomography (OCT), and OCT angiography (OCTA). OCT and OCTA were performed using a custom-built 780 nm spectral-domain engine and ophthalmic scanner [15,22]. Testing was obtained at baseline and prior to each week's injection, and then 1 week after the final (third) injection (immediately prior to sacrificing the rabbit and harvesting the eye).

For ERG studies

- Rabbit eyes were dilated with 0.5% proparacaine hydrochloride, 1% cyclopentolate hydrochloride, and 1% tropicamide ophthalmic solutions.
- Rabbits were dark adapted for at least 1 h by covering their cages.
- After at least 1 h of dark adaptation, lights were turned off in the procedure room, and red lights were turned on in the room. Please note that no regular room lights or regular flashlights can be on during the procedure. All "light leaks" around doors must be kept covered.
- Anesthesia was induced with ketamine and xylazine. Isofluorane is avoided because it may affect ERG recordings.
- Rabbits were placed on the stage for the ERG instrument and wrapped in towels to maintain body temperature.
- Recording and ground subdermal eletrodes were placed in the appropriate positions.
- Goniovisc was placed on the contact lens electrode, and the contact lens electrode was then placed in the rabbit's eye.
- A Faraday cage was placed over the rabbit on the stage of the instrument.
- An electrode test was conducted according to the manufacturer's instructions to ensure that the electrodes were working correctly.
- ERG (OcuScience, Henderson, NV) was performed according to the modified International Standard for Clinical Electrophysiology of Vision (ISCEV) protocol for rabbits [22].

Photography and intravenous flouorescein angiography

- Isoflurane anesthesia is used for photography.
- Topical anesthetic drops (proparacaine) and dilating drops (cyclopentolate and/or tropicamide) are instilled in the rabbit eye(s). Atropine is generally not used in rabbits because they possess the enzyme atropinase.
- Photographs are taken with a Pictor Plus fundus camera (Volk, Mentor, OH).
- After completing the regular fundus photography, a rapid bolus injection of fluorescein at a dose of 10–25% in a volume up to 2 mL was given, and serial images were immediately captured with the Pictor Plus fundus camera with the FA attachment. The general dosing used was 7.7 mg/kg body weight, similar to what is used for young children.

Optical Coherence Tomography (OCT) and OCT angiography (OCTA)

- This is performed under isoflurane anesthesia, and following dilation and regular fundus photography and fluorescein angiography as described above.
- A recirculating water blanket was placed around the rabbit to maintain body temperature, and the rabbit was placed on a stand for the imaging.
- Imaging was performed using a custom-built 780 nm spectral-domain engine and ophthalmic scanner [23], as has been described previously.

Histologic sample preparation

- After rabbits were sacrificed, the eves were harvested and fixed in Davidson's solution for 48-72 h. Small holes were created on opposite sides of the eye using a dermal punch to improve penetration of the fixative evenly. The eye was then embedded in paraffin.
- Histologic sections were evaluated by an experienced veterinary pathologist.

Statistical analyses of rabbit ERG data

ERG was performed in order to measure the retinal function of rabbits before and after intravitreal drug treatment. Retinal responses to scotopic 100 mcd flashes, scotopic 3000 mcd flashes, scotopic 10,000 mcd flashes, photopic 3000 mcd flashes, and 30-Hz flicker flashes were conducted, according to the modified ISCEV protocol [22]. For each specific ERG test, the response values of a-wave and b-wave amplitudes, and a- and b-wave implicit times were recorded. For assessment of toxicity in individual rabbits, toxicity was defined as a 25% reduction in ERG amplitude or a 25% prolongation of implicit time for each test and each response variable [9,15].

The percentage change rate is calculated by the change in ERG value (amplitude or implicit time) from baseline to the end of time point (or between pre-and post-treatment) divided by the ERG value at baseline, i.e. $(y_{end} - y_{baseline})/y_{baseline} \times 100\%$ where y_{end} is the ERG value at the end of time point, and $y_{baseline}$ is the ERG value at baseline for a given rabbit. Thus, a rabbit experiences toxicity if at least one ERG response meets the toxicity criteria (the percent change rate of amplitude $\leq -25\%$ or the percent change rate of implicit time $\geq 25\%$).

We are interested in the treatment group effect over weeks (or time) on ERG amplitude or implicit time (response variables). In a linear model, we would model this as response=treatment group + week + treatment \times time+ ε . The treatment group is a factor variable, the week is a numeric variable (it would be 0 and 1 if the week is only for pre-treatment and post-treatment), and error term ε is a random (independent) variable representing the deviation of a value of ERG from the predicted value. In the study, the same rabbit's ERG values are usually more similar to each other than those from the different rabbits. Since the difference within a rabbit's ERG recordings are interdependent (correlated), this would violate the model assumption of independence. A linear model was extended to account for correlation among observations within the same rabbit by incorporating random effects, additional error term b (also noted as (1|rabbit)). The updated formula will be response = treatment + week + treatment × time + $+\varepsilon$, *i.e.*, a mixed-effect model with fixed effect and the random intercepts for each rabbit (letting the intercept vary by rabbit). Given a specific ERG test and a response variable, assume that the ERG value (\mathbf{y}_i) is defined as

$$\mathbf{y}_i = \mathbf{X}_i \boldsymbol{\beta} + \mathbf{z}_i b_i + \varepsilon_i$$
, rabbit $i = 1, 2, \dots, s$

 $y_i: (n_i \times 1)$ vector of response variables measured on rabbit i and n_i is the number of repeated measurements for rabbit i.

 β : ($p \times 1$) vector of parameters (fixed effects). We have one parameter for fixed intercept effect, $(l_t - 1)$ parameters for the treatment group, one parameter for the week, and $(l_t - 1)$ parameters for the interaction between treatment groups and week where l_t is the number of levels for the treatment group. The number of parameters $p = 1 + (l_t - 1) + 1 + (l_t - 1)$.

 X_i : $(n_i \times p)$ known matrices of full rank.

 \mathbf{z}_i : $(n_i \times 1)$ known matrices of full rank, $\mathbf{z}_i = (1, 1, ..., 1)'$

- b_i : a random intercept (random effects), $b_i \sim N(0, \sigma_b^2)$ where σ_b^2 is the between-rabbit variability. ε_i : $(n_i \times 1)$ random error (within-rabbit) term, $\varepsilon_i \sim N(0, \sigma^2 I)$ where σ^2 is the within-rabbit variability.

 β , σ_b^2 , and σ^2 can be estimated with either the maximum likelihood (ML) method or restricted maximum likelihood (REML) method using the *lme* function from the *nlme* library in **R** [24,25].

After the model is fitted, the correlation among observations within the same rabbit can be calculated by the ratio of the estimate between-rabbit variance $(\hat{\sigma}_b^2)$ to the estimated total variance $(\hat{\sigma}_h^2 + \hat{\sigma}^2)$, named the intraclass correlation (ICC). ICC represents the proportion of the total variance in ERG data that is accounted for by the rabbit. The adjusted mean values (least-squares means) with a 95% confidence interval for each treatment group and each week can be estimated using the *emmeans* function and *summary* function from the *emmeans* library [26]. If data are fitted on the original scale, the adjusted mean values are estimated with type='lp' in *summary* function. Instead, use type='response' for the inverse transformation. Next, *emtrends* function is used to estimate and test each treatment group's trend with the Wald test. The var in *emtrends* function should be a numeric predictor instead of a factor variable. The adjusted percentage change rate is calculated by the change in the adjusted average ERG value from baseline to the end of time point (or the adjusted mean of the difference between pre-and post-treatment value) divided by the adjusted ERG value at baseline, *i.e.*, $(\hat{y}_{end} - \hat{y}_{baseline})/\hat{y}_{baseline} \times 100\%$ where \hat{y}_{end} is the adjusted average ERG value at the end of the time point and $\hat{y}_{baseline}$ is the adjusted average ERG value at baseline. Thus, the toxicity for each treatment group (dosages) was defined when (1) the difference between pre- and post-treatment is statistically significant from the trend test and (2) the adjusted percentage change rate meets the toxicity criteria (the adjusted percent change rate of amplitude $\leq -25\%$ or the adjusted percent change rate of implicit time $\geq 25\%$).

For evaluating the trend difference among the treatment groups, the *contrast* function from *emmeans* package with method='pairwise' (or *pairs* function) is applied. The Bonferroni correction was used with a multiple comparisons procedure to control the experiment-wise type I error rate at 5% for each specific ERG test and each response value. To better meet the normality assumption, data is transformed on a natural log scale or a square root scale as needed. All tests were two-sided and were performed using R version 3.6.3.

Assessment of efficacy for vitreous seeds in vivo in rabbits

This orthotopic xenograft vitreous seed rabbit model was originally based on the work of Kang and Grossniklaus [27], and was further developed by us subsequently [9,21].

- Cyclosporine injections were begun 3 days prior to the injection of the cells at a dose of 15 mg/kg subcutaneously daily, and continued throughout the experiment at a dose of 15 mg/kg for 14 days and then 10 mg/kg for the remainder of the experiment.
- Anesthetics drops (proparacaine) and dilating drops (tropicamide and/or cyclopentolate) were placed in the rabbit eye(s).
- Anesthesia was induced with isoflurane.
- Betadine was instilled on the ocular surface and a lid speculum was placed.
- If an anterior chamber tap was to be performed (*e.g.*, for biomarker analysis), then it was performed at this point, using a 30G needle on a 1 cc syringe. We generally favor performing a pre-injection aqueous tap to reduce the intraocular pressure and prevent efflux of cells (or drug).
- 1 million WERI-Rb-1 cells were resuspended in 100 µL 1X PBS and injected into the vitreous of either one or both eyes of the rabbit using a 30G needle on a 1 cc syringe, as we have described previously [9,21].
- The rabbit eye(s) were rinsed with sterile balanced salt solution.

For weekly injections of drug, the same protocol as above was repeated, except that 100µL of drug (or saline control) was injected instead of cells each time. Generally, the retinoblastoma cells were allowed to grow into vitreous seeds for 2 weeks prior to beginning treatments [21]. Similarly, after the final intravitreal drug injection, we tend to wait 2 weeks before sacrificing the rabbits and harvesting the eye(s), since there is evidence that the effect of many intravitreal drugs may persist for 2 weeks (or longer) [9].

Quantification of vitreous seeds and assessment of efficacy

- After the rabbit is euthanized, the eye(s) are removed *in toto*, being careful not to cut the sclera on the back side of the globe.
- Trim off the conjunctiva/muscles/episcleral tissue/fat with a scissors.

- Use a #11 blade to make a small incision in the peripheral cornea (~1-2 clock hours near the peripheral cornea just anterior to the limbus).
- Complete removing the cornea with a sharp-pointed scissors (and toothed forceps).
- Use the scissors to cut out the iris circumferentially near it's root.
- Use a toothed forceps to grasp and pull out the lens.
- Use a small, toothed forceps to grab the ciliary body (and any residual peripheral iris root) and pull out the vitreous body *in toto* (being careful not to grab the retina). It usually comes out within the "bag" of the vitreous hyaloid.
- The vitreous body is then transferred to a six well plate. The ciliary body "ring" is carefully removed from the well, taking care not to disturb the vitreous seeds/tumor cells, which are easily visible within the vitreous in the well. Visual contrast can be improved by performing this over top of a blue sheet, which highlights the white tumor seeds. If there is any concern that there is residual (liquid) vitreous remaining within the scleral shell, it can be added to the well by pouring, taking care not to disturb the retina.
- 2 mL hyaluronidase (1 mg/mL stock in 1X PBS), 0.4 mL collagenase (10 mg/mL stock in 1X PBS), and 0.6 mL RPMI (no phenol red) are added to the well with the vitreous.
- The plate is placed in a 37 °C cell culture incubator overnight.
- The next day the solution from each well is placed in a 50 mL conical tube and spun down. The resulting supernatant is aspirated.
- The cell pellet is resuspended in 1X PBS and counted using trypan blue.
- Hyaluronidase is from Sigma (catalog# H3506), and collagenase is from Sigma (catalog# C0130).

Statistical analyses of rabbit efficacy data

For evaluating efficacy of a given drug

For evaluating the efficacy of a treatment, each rabbit's right eye is treated with serial injections of the study drug and the contralateral left eye is treated with saline as an internal control [9,28] The cell number was measured in the experiment. In order to reduce the variability of the cell number variable, cell number is transformed on a natural log or a square root scale. The drug effect can be evaluated by the difference in cell number on the transformed scale between the correlated samples (left saline-treated eyes and right drug-treated eyes) using the paired *t*-test.

For comparing efficacy between two different drugs

General Considerations:

There are two different statistical methodologies that we use for comparing relative efficacy of two different drugs (for example, to compare the efficacy of a new drug to standard-of-care melphalan). We generally favor presenting the first method in publications, because it is conceptually easier to understand, and the graphs that are generated are more intuitive to read and understand. The mixed effect modeling involved in the second method is not intuitive to most readers, but is probably a more "correct" way to view the data from a statistical standpoint. From a practical standpoint, the two yield almost identical results.

Method #1:

For comparing the efficacy *between* the different drug treatment groups, a relative reduction of cell count (%) is calculated by the difference in cell count from left (control) eye to right (treated) eye divided by the cell count from left (control) eye. The relative reduction of cell number was analyzed to compare the two independent groups using the Welch two-sample *t*-test.

Method #2:

For comparing the efficacy between the different treatment groups, the analysis was conducted using the mixed-effect model to account for the correlations of cell counts between the left (saline-treated control) eye and right (drug-treated) eye from the same rabbit. The treatment group and eye variables will be fixed effect in the model to use *lme* function. The *emmeans* function and the *summary* function are used to estimate the adjusted mean with a 95% confidence interval given by the treatment group and eye. For trend estimation, the reduction of cell counts from the right (drug-treated) eye to the left (saline-treated control) eye by treatment group were estimated using the

emtrends function. Var in the *emtrends* function should be a numeric predictor (0 for the left eye and 1 for the right eye). The difference in efficacy between the treatment group was compared using the *pairs* function. Finally, the adjusted percentage of reduction rate can be obtained by the adjusted average change from the left (control) eye to right (treated) eye divided by the adjusted cell count from the left (comparator treatment) eye for each treatment group. To better meet the normality assumption, cell count was analyzed on a natural log scale. Standard residual analysis was evaluated. All tests were two-sided and were performed using R version 3.6.3.

Validation of the above method and protocol

We have used the above protocol to evaluate the efficacy and toxicity of various potential alternative chemotherapy agents (*e.g.* topotecan [28]) and targeted inhibitors (*e.g.* belinostat) for intravitreal injection, and have determined an ideal injection dose in rabbits (and in humans, accounting for the differences in eye vitreous volume between rabbits and humans), as well as to directly compare various formulations of melphalan [9].

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

ABD has a patent with Vanderbilt University. ABD has received research funding from Spectrum Pharmaceuticals (now Acrotech Biopharma) for an investigator-initiated study related to studies in which the presented methods have been previously used.

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