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# Evaluation of intravitreal topotecan dose levels, toxicity and efficacy for retinoblastoma vitreous seeds: a preclinical and clinical study

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## ABSTRACT

**Background** Current melphalan-based intravitreal regimens for retinoblastoma (RB) vitreous seeds cause retinal toxicity. We assessed the efficacy and toxicity of topotecan monotherapy compared with melphalan in our rabbit model and patient cohort.

**Methods** Rabbit experiments: empiric pharmacokinetics were determined following topotecan injection. For topotecan (15 µg or 30 µg), melphalan (12.5 µg) or saline, toxicity was evaluated by serial electroretinography (ERG) and histopathology, and efficacy against vitreous seed xenografts was measured by tumour cell reduction and apoptosis induction. Patients: retrospective cohort study of 235 patients receiving 990 intravitreal injections of topotecan or melphalan.

**Results** Intravitreal topotecan 30 µg (equals 60 µg in humans) achieved the IC<sub>90</sub> across the rabbit vitreous. Three weekly topotecan injections (either 15 µg or 30 µg) caused no retinal toxicity in rabbits, whereas melphalan 12.5 µg (equals 25 µg in humans) reduced ERG amplitudes 42%–79%. Intravitreal topotecan 15 µg was equally effective to melphalan to treat WERI-Rb1 cell xenografts in rabbits (96% reduction for topotecan vs saline (p=0.004), 88% reduction for melphalan vs saline (p=0.004), topotecan vs melphalan, p=0.15). In our clinical study, patients received 881 monotherapy injections (48 topotecan, 833 melphalan). Patients receiving 20 µg or 30 µg topotecan demonstrated no significant ERG reductions; melphalan caused ERG reductions of 7.6 µV for every injection of 25 µg (p=0.03) or 30 µg (p<0.001). Most patients treated with intravitreal topotecan also received intravitreal melphalan at some point during their treatment course. Among those eyes treated exclusively with topotecan monotherapy, all eyes were salvaged.

**Conclusions** Taken together, these experiments suggest that intravitreal topotecan monotherapy for the treatment of RB vitreous seeds is non-toxic and effective.

## INTRODUCTION

Vitreous seeds have historically been the most difficult-to-treat aspect of intraocular retinoblastoma (RB).<sup>1 2</sup> RB tumours with vitreous seeds are those least likely to be salvaged with radiation<sup>3</sup> or intravenous chemotherapy.<sup>1 2 4 5</sup> Newer

approaches to delivering chemotherapy, including intra-arterial chemotherapy (IAC)<sup>6</sup> and direct intravitreal injection of chemotherapy,<sup>7 8</sup> have partially overcome the treatment-resistant nature of vitreous seeds and have improved globe salvage rates for RB.<sup>9 10</sup> However, the primary chemotherapeutic agent used in both IAC and intravitreal chemotherapy is melphalan, which has been associated with retinal toxicity.<sup>8 11–13</sup> Thus, while intravitreal melphalan may be effective, retinal functional loss is common.<sup>12 14</sup> Furthermore, the toxicity is dose-dependent<sup>15</sup> and worsens with each subsequent melphalan injection delivered.<sup>12 13 16</sup>

Recently, topotecan has been explored as an alternative chemotherapy agent, both by intravenous,<sup>17</sup> intra-arterial<sup>18</sup> and intravitreal<sup>19 20</sup> routes. Preliminary laboratory and clinical evidence suggests that topotecan may be less toxic than current standard-of-care melphalan.<sup>20 21</sup> However, it is unclear if a non-toxic dose of topotecan is clinically effective.<sup>21</sup> Further, it is unclear just how effective topotecan is as monotherapy, as many centres have generally used it in combination with melphalan,<sup>22</sup> or have been quick to re-add melphalan back into the regimen if topotecan monotherapy appeared to not achieve adequate tumour control.<sup>20</sup> Likewise, the optimal dose of topotecan that best balances efficacy with toxicity as intravitreal monotherapy has not been established.

We recently developed a rabbit xenograft model of RB with vitreous seeds and retinal tumours, which we have used to study the toxicity of IAC, exploring various different IAC drugs.<sup>23 24</sup> We have also previously described a complete platform to assess functional and structural retinal toxicity associated with local delivery of various chemotherapeutic agents.<sup>11</sup> Here, we use this rabbit model<sup>23</sup> and this toxicity evaluation platform,<sup>11</sup> to determine the dose of intravitreal topotecan which is effective and non-toxic when delivered as monotherapy. We then corroborate this evidence of non-toxicity with our clinical experience treating RB patients with vitreous seeds with intravitreal topotecan.

## METHODS

### Statement of research ethics

All animal experiments adhered to the Association for Research in Vision and Ophthalmology



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Statement on Animal Use and were performed under the auspices of the Vanderbilt Institutional Animal Care and Use Committee.

### Intravitreal topotecan pharmacokinetics

New Zealand white rabbits (2.8–3.0 kg) were used for all studies. For pharmacokinetic experiments, a 20-gauge valved vitrectomy cannula was inserted 2–3 mm behind the limbus. One micro-gram topotecan hydrochloride was injected on the opposite side 2–3 mm behind the limbus into the vitreous cavity. Serial vitreous taps were performed through the valved cannula at 30 min, 1 hour, 2 hours, 4 hours and 6 hours. Use of a valved cannula-maintained eye stability and prevented efflux of vitreous contents during manipulations.<sup>25</sup> Vitreous samples were immediately placed on dry ice and then stored at  $-80^{\circ}\text{C}$  until drug levels were measured.

Vitreous samples were thawed, an internal carbamazepine standard was added and samples were diluted with blank plasma, then deproteinised with acetonitrile. Samples were analysed on a Thermo Scientific TSQ Quantum Ultra mass spectrometer interfaced to a Waters Acquity UPLC system, using methodology we have reported previously.<sup>23</sup>

Topotecan concentrations were averaged across rabbits at each time point. The resulting mean time-concentration data from each matrix were analysed via non-compartmental analysis (Phoenix WinNonlin V.6.4, Pharsight/Certara USA, Princeton, New Jersey, USA) to determine pharmacokinetic parameters, including half-life.

### In vitro determination of dosing

Human WERI-Rb1 RB cells ( $5 \times 10^3$ ) were plated in 96-well plates in the presence of various concentrations of topotecan for 16 hours (five half-lives as determined through the above pharmacokinetic experiments). Topotecan-containing media was then removed, and fresh media added. After 7 days, the Cell-Titer Blue assay (Promega, Madison, Wisconsin, USA) was used to count live cells. Survival curves were graphed with GraphPad, and the  $\text{IC}_{90}$  was calculated.

Using the pharmacokinetic parameters determined above, we calculated<sup>25</sup> the dose of topotecan that would need to be injected into the eye to achieve the  $\text{IC}_{90}$  in the vitreous on the opposite side of the eye for a duration of five half-lives.

### Assessment of efficacy of intravitreal topotecan for vitreous seeds in rabbits

Figure 1A depicts our experimental design. RB vitreous seeds were created by intravitreal injection of 1 000 000 WERI-Rb1 cells in

100  $\mu\text{L}$  saline in both eyes of cyclosporine-immunosuppressed rabbits, as we have described previously.<sup>23 25</sup> After 2 weeks of growth, the right eyes received three weekly injections of either 15  $\mu\text{g}/100 \mu\text{L}$  topotecan or 12.5  $\mu\text{g}/100 \mu\text{L}$  melphalan, while all left eyes received 100  $\mu\text{L}$  saline.

Two weeks after the final injection, all rabbits were euthanised, and the eyes were removed. For five rabbits, the vitreous of each eye was harvested and digested in 0.5 mg/mL hyaluronidase and 1 mg/mL collagenase overnight at  $37^{\circ}\text{C}$ . Live cells were counted by direct microscopy using trypan blue stain. In four additional rabbits from each treatment group, the entire eyes were submitted for histopathology (two rabbits after receiving three injections and two rabbits after receiving a single injection).

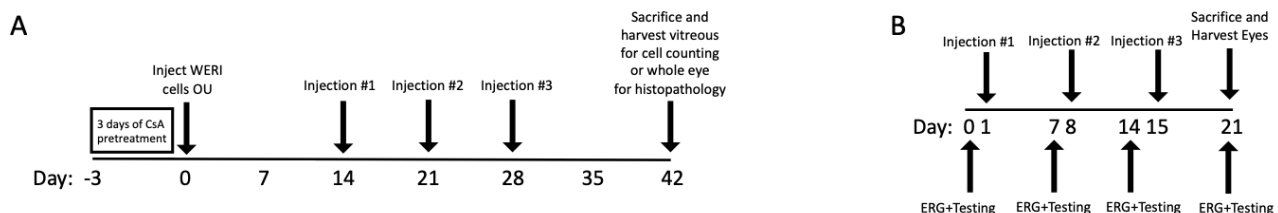
### Assessment of ocular toxicity of intravitreal topotecan in rabbits

Four cohorts ( $n=4-6$  rabbits/cohort) received either topotecan 30  $\mu\text{g}$  (the calculated  $\text{IC}_{90}$ ), topotecan 15  $\mu\text{g}$  (half the calculated  $\text{IC}_{90}$ ), saline (control) or melphalan 12.5  $\mu\text{g}$  (current standard-of-care).<sup>26</sup> In all rabbits within a given cohort, the right eyes received three injections, one injection per week, of the same drug/dose. Figure 1B depicts our experimental design. Electroretinography (ERG; OcuScience, Henderson, Nevada, USA) was performed according to the modified International Standard for Clinical Electrophysiology of Vision protocol for rabbits.<sup>27</sup> Intravitreal injections were performed weekly, and always within 1 day following testing. After euthanasia, eyes were harvested and fixed in Davidson's solution.

Toxicity was defined for every ERG parameter, using our previously published definition.<sup>11 25</sup> Briefly, toxicity was deemed significant for a given dose in a rabbit group if there was a 25% reduction in average ERG amplitude, or a 25% prolongation of average implicit time comparing the post-treatment parameter values after three injections with the pretreatment values, if the difference was statistically significant.

### Ocular toxicity and efficacy of intravitreal topotecan versus melphalan in patients

Medical records of all patients treated with intravitreal injections at Memorial Sloan-Kettering Cancer Center and Vanderbilt University Medical Center were reviewed. Patients receiving intravitreal topotecan were identified. A second cohort of all patients receiving melphalan as monotherapy were included as a comparator group. Injection number, drug and dose were recorded. ERGs were performed using a previously published,



**Figure 1** Experimental design of efficacy and toxicity experiments in the rabbit model. (A) Efficacy experiments. Following 3 days of cyclosporine immunosuppression (which also continued throughout the course of the experiment), WERI-Rb1 cells were injected into the vitreous of both eyes of New Zealand white rabbits. After 2 weeks of growth, once-per-week injections of drug (topotecan 15  $\mu\text{g}$  or melphalan 12.5  $\mu\text{g}$ ) were given into the right eye (OD), and saline into the left eye (OS). Imaging was obtained and rabbits were sacrificed 2 weeks after the third injection. (B) Toxicity experiments. Drug (topotecan, melphalan or saline, depending on the cohort) was injected into the right eye (OD) of New Zealand white rabbits once per week for three consecutive weeks. One day prior to each injection, functional testing was obtained (see the Methods section). One week following the final (third) injection, testing was again performed, and then the rabbits were sacrificed and globes harvested for histopathological evaluation. ERG, electroretinography. CsA=cyclosporine A.

and validated, abbreviated ERG protocol.<sup>28–30</sup> For efficacy, we included all treated patients for which complete records were available. Our toxicity analyses included only those monotherapy injections for which this ERG protocol was performed prior to the intravitreal injection, as well as subsequent to the injection, and patients who also received concomitant IAC between the two ERGs were excluded. Ocular and systemic adverse events, as well as clinical outcomes, were recorded.

### Statistical analyses of rabbit and human efficacy and ERG data

For univariate analysis to compare toxicity in patients, the Wilcoxon signed-rank test was used. For multivariable analysis, to evaluate the toxicity of each drug at different dosages, a linear mixed-effects model was fitted with treatment groups and the repeated measurements (pre or post) for each parameter and each test. Using model-based (least-square) means, the average adjusted change from pretreatment versus post-treatment and the difference in change between different treatment groups (difference-of-differences) were estimated and compared with the Wald test. Our predefined definition of toxicity (see earlier) was used in the rabbit analyses. Data were transformed to better meet normality assumptions and adjusted for heteroscedasticity when necessary. To account for multiple comparisons, Bonferroni-adjusted p values were reported (two-tailed), with adjusted p values less than 0.05 considered statistically significant. The analyses were performed using R V.3.6.3 including packages ‘nlme’ and ‘emmeans’. For experiments in rabbits, ‘pre’ and ‘post’ were defined before and after the three injections. For human experiments, because of the variability between dosing and interval of injections, ‘pre’ and ‘post’ were defined on a ‘per injection’ basis without minimum reduction limits, and inter-eye/intra-patient and intra-eye correlations were taken into account in our modelling.

For efficacy experiments in rabbits comparing paired right eyes receiving topotecan (or melphalan) and contralateral left eyes receiving saline, the paired t-test was used. Relative reduction of cell counts was analysed to compare the difference between two independent groups (topotecan and melphalan rabbit cohorts) using Welch two sample t-test.

## RESULTS

### In vivo topotecan pharmacokinetics and in vitro determination of expected effective in vivo dose

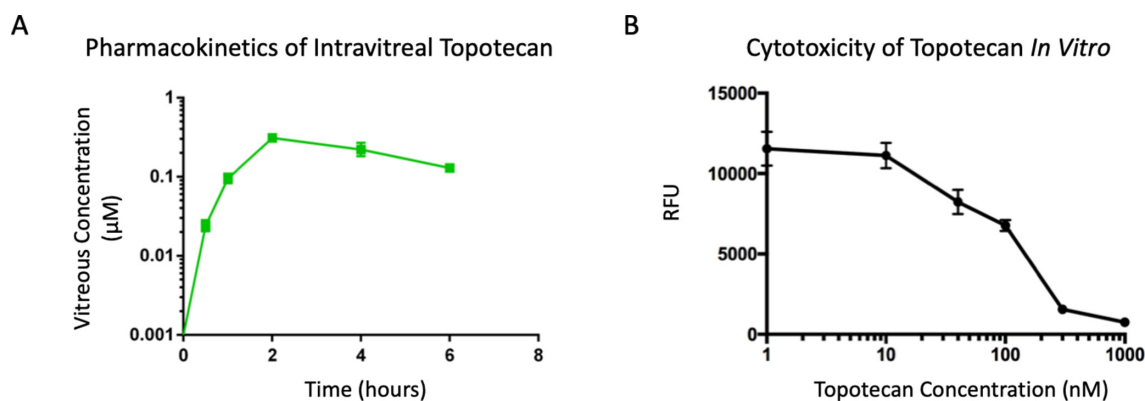
Peak concentration at the opposite side of the eye was achieved at 2 hours postinjection (figure 2A). The average  $C_{max}$  at the opposite side of the eye was  $0.31 \mu\text{mol/L}$ . However, the theoretical  $C_{max}$  (calculated as  $1 \mu\text{g}/1.4 \text{ mL}$  rabbit vitreous volume) is  $1.56 \mu\text{mol/L}$ . Therefore, compared with the theoretical  $C_{max}$ , the actual empiric  $C_{max}$  at the opposite side of the eye achieved 2 hours after injection was  $\sim 20\%$  of expected, likely due to rapid efflux during this period of slow diffusion of topotecan across the vitreous. The half-life of topotecan in the rabbit eye was 3.27 hours. We therefore exposed WERI-Rb1 human RB cells to various doses of topotecan for five vitreous half-lives ( $\sim 16$  hours total) and measured live cells 7 days later. The  $IC_{90}$  was  $300 \text{ nM}$  (figure 2B). We calculated that we would need to inject  $30 \mu\text{g}$  topotecan to sustain this  $IC_{90}$  concentration for 16 hours (five half-lives) at the opposite side of the rabbit eye.

### Relative efficacy of intravitreal topotecan versus melphalan to treat RB vitreous seeds in vivo in rabbits

Three weekly injections of  $15 \mu\text{g}$  topotecan killed 96% of vitreous seed tumour cells, compared with saline-treated contralateral eyes ( $p=0.004$ , figure 3). Three weekly injections of  $12.5 \mu\text{g}$  melphalan (corresponding to the clinically used dose of  $25 \mu\text{g}$  in patients) killed 88% of cells, compared with saline-treated contralateral eyes ( $p=0.004$ ; topotecan vs melphalan:  $p=0.15$ , figure 3). For additional rabbits in each cohort, the entire eyes were harvested and submitted for histopathology. Residual RB cells in the topotecan-treated eyes were TUNEL positive, suggesting that the  $\sim 4\%$  of ‘remaining’ cells counted in the vitreous seed quantitation assay were likely in the process of dying as well.

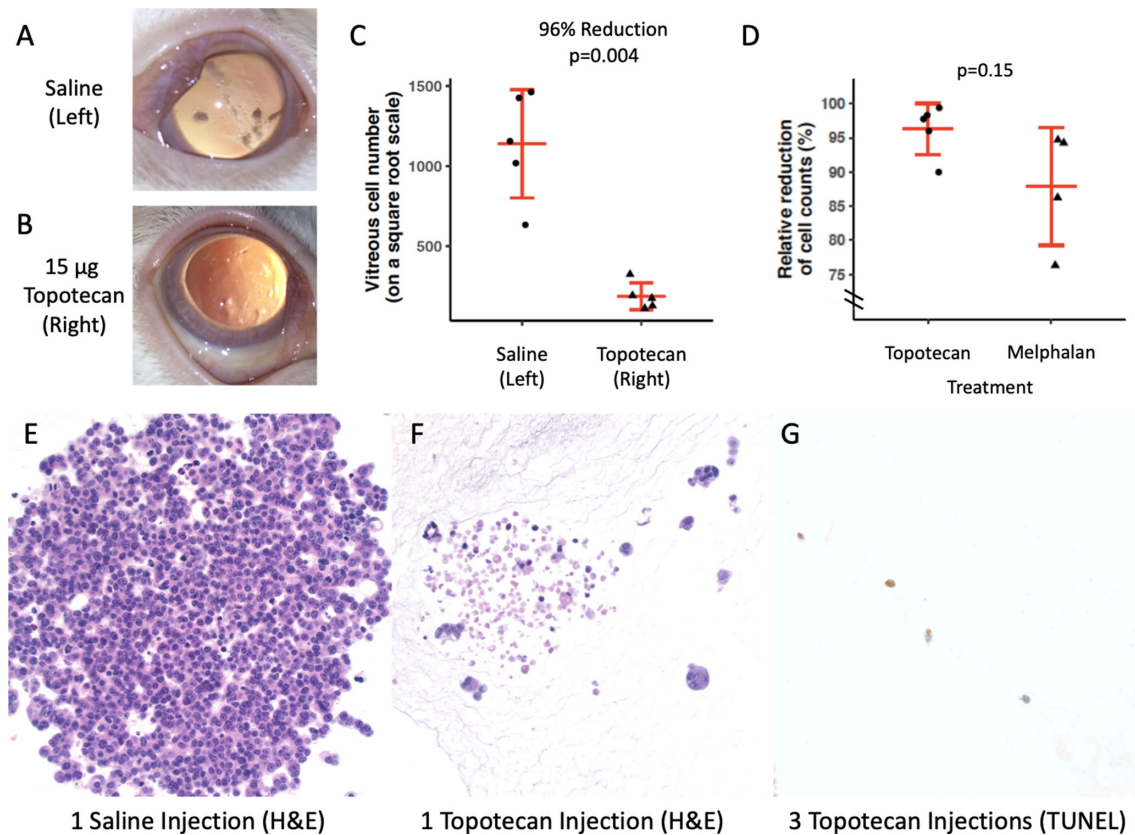
### Toxicity of various doses of intravitreal topotecan compared with melphalan in rabbits

While there was no worsening of ERG parameters in the saline control group, melphalan caused significant worsening of almost all ERG parameters, with reductions in ERG amplitudes between 42% and 79% (figure 4A). These ERG changes



**Figure 2** Pharmacokinetics of intravitreal topotecan in the rabbit eye and dose-dependent survival curves of retinoblastoma cells to transient exposure to topotecan in vitro. (A) Pharmacokinetic curve in the rabbit vitreous following intravitreal injection of  $1 \mu\text{g}$  of topotecan hydrochloride. Sampling was performed through a valved vitrectomy cannula inserted on the contralateral side of the eye relative to the injection site, and all sampling was performed on the far side of the vitreous relative to the injection site. The use of a valved vitrectomy cannula helped to maintain eye stability and prevent leakage throughout the experiment. (B) CellTiter Blue survival curve in human WERI-Rb1 cells exposed in vitro to various doses of topotecan hydrochloride for 16 hours (equal to five vitreous half-lives). Surviving cells were measured at the 7-day time-point. The  $IC_{90}$  can be found to correspond to  $300 \text{ nM}$  topotecan hydrochloride for this length of exposure. RFU=relative fluorescence units.





**Figure 3** Efficacy of intravitreal topotecan against retinoblastoma vitreous seeds in a rabbit xenograft model. One million WERI-Rb1 human retinoblastoma cells were injected into the vitreous of both eyes of cyclosporine-immunosuppressed rabbits. Following 2 weeks of growth, the eyes were given three weekly injections of the intravitreal drug (right eye) or intravitreal saline (left eye). (A–B) Residual vitreous seeds 2 weeks following the final (third) injection, showing (A) significant large seeds in a representative left eye, and (B) complete clinical resolution of the vitreous seeds in a representative topotecan-treated right eye. (C) Quantification of residual live vitreous seeds 2 weeks after the third injection of topotecan (right eyes) or saline (left eyes), showing reduction in live tumour cells with topotecan 15  $\mu$ g. Note the scale is a square root scale and is not linear. (D) Comparison of relative reduction with topotecan versus melphalan (compared with their respective saline-treated contralateral eyes), demonstrating no statistically significant difference between the two treatments. Note the abbreviated Y-axis only extends from 75% to 100%. (E) H&E stain of a vitreous seed 2 weeks after a single saline injection in the control eye. (F) H&E stain of a vitreous seed 2 weeks after *one single injection* of 15  $\mu$ g topotecan, showing significant cell death in most cells. (G) An eye treated with 3 weekly injections of 15  $\mu$ g topotecan. TUNEL staining shows that all remaining cells are in the process of dying. Taken together with our quantitative cell counting experiments, which showed a 96% reduction in tumour cells with three weekly injections of topotecan, this indicates that even the ‘surviving’ 4% of cells were actually in the process of dying as well. H&E=hematoxylin and eosin.

occurred in every rabbit within the cohort, with a median of 9 (IQR: 7–9, out of 18) parameters affected per rabbit. Similarly, implicit times were prolonged. Histopathology demonstrated severe atrophy affecting all retinal layers, worst near the injection sites (figure 4B–D).

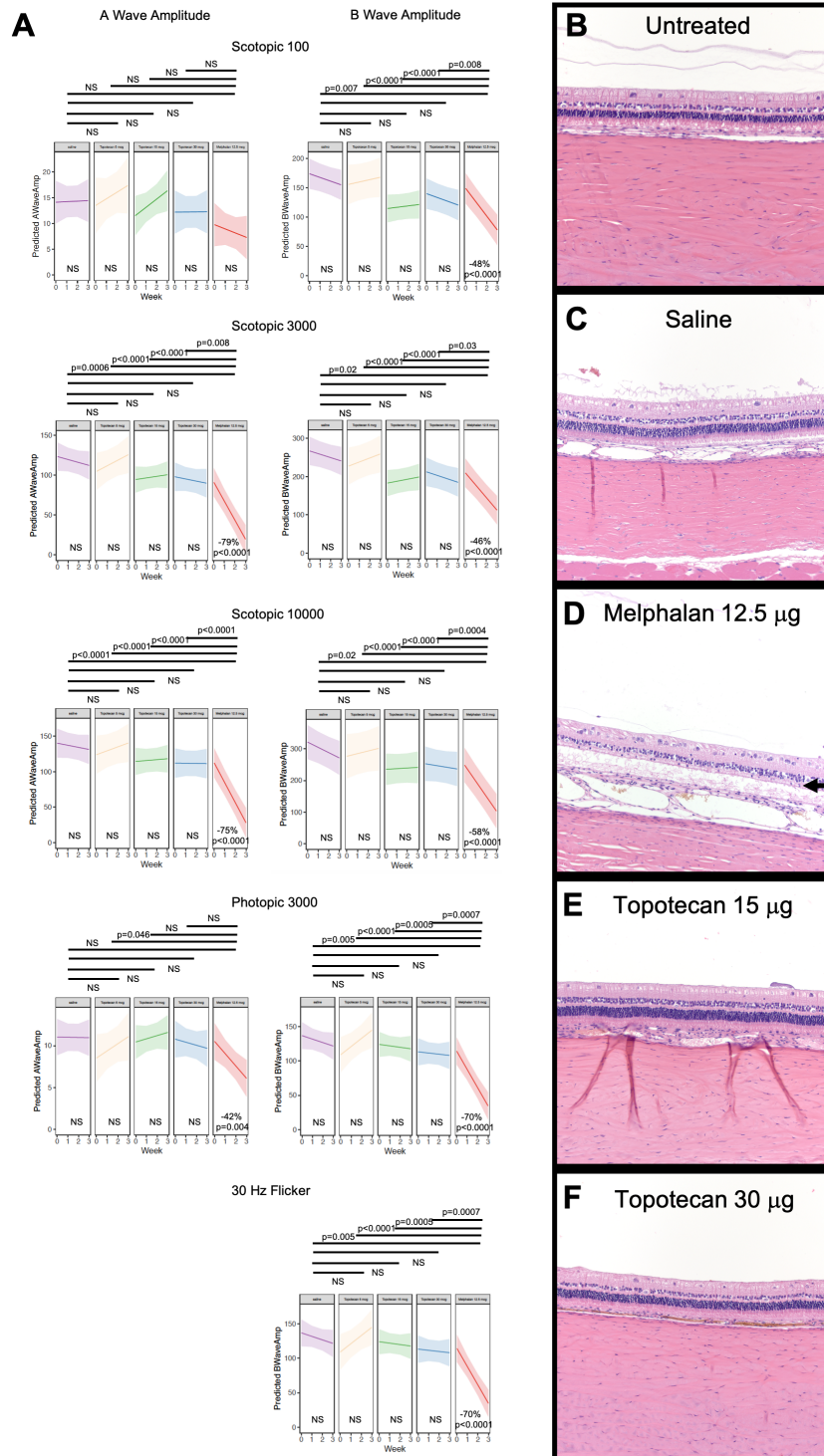
In contrast, rabbits in the 15  $\mu$ g and 30  $\mu$ g topotecan cohorts did not experience any statistically or clinically meaningful worsening of ERG parameters (figure 4A). Even at twice the clinically effective dose, multiple repeated weekly intravitreal topotecan injections did not cause retinal toxicity. No other signs of toxicity were observed on clinical examination, and histopathology showed none of the retinal damage that was seen in the melphalan-treated groups, with retinas of eyes treated with topotecan being histologically indistinguishable from saline-treated control eyes (figure 4B–F).

#### Comparative toxicity in patients receiving intravitreal topotecan at various doses compared with melphalan

In 41 patients, 108 intravitreal injections of topotecan were given to 42 eyes. Of these 108 injections, 48 injections consisted

of topotecan as intravitreal monotherapy, at dosages of either 30  $\mu$ g (18 injections), 20  $\mu$ g (29 injections) or 10  $\mu$ g (one injection). In general, the lower dose of 20  $\mu$ g was used until ~2017, and 30  $\mu$ g was used beginning in mid-2017, when it was felt that the efficacy-versus-toxicity balance warranted an increase in dose (the single treatment with 10  $\mu$ g was given in 2014). Preinjection and postinjection ERG data were available for 40 topotecan injections. Nine ‘undetectable’ pretreatment ERGs (<5  $\mu$ V) were excluded. Six additional injections were excluded because they were still receiving concomitant IAC. Ultimately, this left 25 topotecan monotherapy injections (from 14 eyes) with evaluable ERGs for analysis (11 at 20  $\mu$ g and 14 at 30  $\mu$ g).

In the comparator group, 882 intravitreal melphalan injections were given to 210 eyes of 194 patients. Of these, 833 injections (205 eyes) consisted of melphalan as intravitreal monotherapy (99 injections at 25  $\mu$ g, 732 injections at 30  $\mu$ g, 2 injections at 40  $\mu$ g). Preinjection and postinjection ERG data using the previously described and validated<sup>28–30</sup> abbreviated clinical protocol were available for 384 injections. Injections were excluded if they were still receiving concomitant IAC, if pretreatment ERGs



**Figure 4** Absence of retinal toxicity with various doses of intravitreal topotecan, compared with melphalan. (A) Retinal function. Electroretinography was performed weekly, 1 day prior to each of the three planned injections, as well as 1 week after the final injection (immediately prior to euthanising the rabbit). 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 recorded. A-wave and B-wave amplitudes, and A-wave and B-wave implicit times were recorded (except for the 30 Hz flicker, for which there is only a B wave). Shaded areas on the graphs represent 95% CIs. No toxicity (see the Methods section for toxicity criteria) was observed for any parameter in the saline-treated control eyes, as well as in the cohorts treated with either 15 µg or 30 µg of topotecan. However, significant toxicity was seen in the cohort of rabbits treated with 12.5 µg of melphalan. Graphs of amplitudes are shown, but similar results were seen for implicit times, as well. For those particular tests where significant toxicity was seen, per cent change and p values for estimates of trend are shown alongside the particular graph. P values of the difference between groups are shown at the top of each graph. (B–F) Histopathology of eyes demonstrating (B) normal retinal architecture in untreated eyes and in (C) the saline-treated eyes. (D) In contrast, eyes treated with 12.5 µg melphalan showed significant retinal atrophy on histopathology (arrow shows the location of loss of outer retinal architecture). Eyes treated with intravitreal injections of (E) 15 µg topotecan, or (F) 30 µg topotecan were histologically indistinguishable from saline-treated (or untreated) eyes. Retinal detachments are artefactual. NS=not significant.

were 'undetectable' ( $<5 \mu\text{V}$ ), or if multiple injections occurred between the preinjection and postinjection ERGs. Thus, the final group included for analysis of toxicity and ERGs included 225 intravitreal melphalan monotherapy injections (66 at 25  $\mu\text{g}$ , 159 at 30  $\mu\text{g}$ ).

In a univariate analysis, patients receiving melphalan experienced a 7.29  $\mu\text{V}$  reduction in ERG amplitude, *per injection* ( $p < 0.001$ ), with no significant difference in the amount of reduction between those receiving 25  $\mu\text{g}$  or 30  $\mu\text{g}$  of melphalan. In contrast, patients receiving topotecan experienced no reduction in ERG amplitude, in either the 20  $\mu\text{g}$  subcohort or the 30  $\mu\text{g}$  subcohort (figure 5).

In a mixed effect model, patients receiving melphalan experienced a 7.55  $\mu\text{V}$  reduction in ERG amplitude, *per injection* ( $p < 0.001$ ), consisting of 7.58  $\mu\text{V}$  reduction per 25  $\mu\text{g}$  injection ( $p = 0.03$ ), and 7.57  $\mu\text{V}$  reduction per 30  $\mu\text{g}$  injection ( $p < 0.001$ ). In contrast, in the mixed effect models, patients receiving topotecan at either 20  $\mu\text{g}$  or 30  $\mu\text{g}$  experienced no reduction in ERG amplitude (figure 5).

### Efficacy of intravitreal topotecan compared with melphalan in patients

There were 23 patients (23 eyes) who were treated with intravitreal topotecan for whom a complete clinical course was available for review (follow-up:  $23.5 \pm 18.8$  months). Of these six, five were treated with 30  $\mu\text{g}$  (all after October 2017), and one was treated with 20  $\mu\text{g}$ . The comparator group consisted of 66 patients (70 eyes) whose entire intravitreal treatment course consisted solely of melphalan monotherapy, receiving a mean of  $4.0 \pm 2.4$  injections (follow-up:  $30.9 \pm 26.0$  months). In this melphalan monotherapy group, seed eradication and globe salvage was achieved in 65/70 (92.9%) of eyes. It is difficult to evaluate the true efficacy of topotecan in this cohort as the majority of patients who received intravitreal topotecan also received intravitreal melphalan at some point during the course of treatment, and so we cannot definitively attribute the seed eradication to the topotecan in those cases. Only six patients received topotecan monotherapy exclusively throughout their intravitreal treatment course, and while the vitreous seeds were successfully eradicated in all of these patients (having received a mean of  $1.8 \pm 0.75$  injections), it is possible that there might have been selection bias whereby the patients with the least significant vitreous tumour burden were most likely to receive only topotecan. Future randomised studies are needed to evaluate the relative efficacy of topotecan versus melphalan.

### DISCUSSION

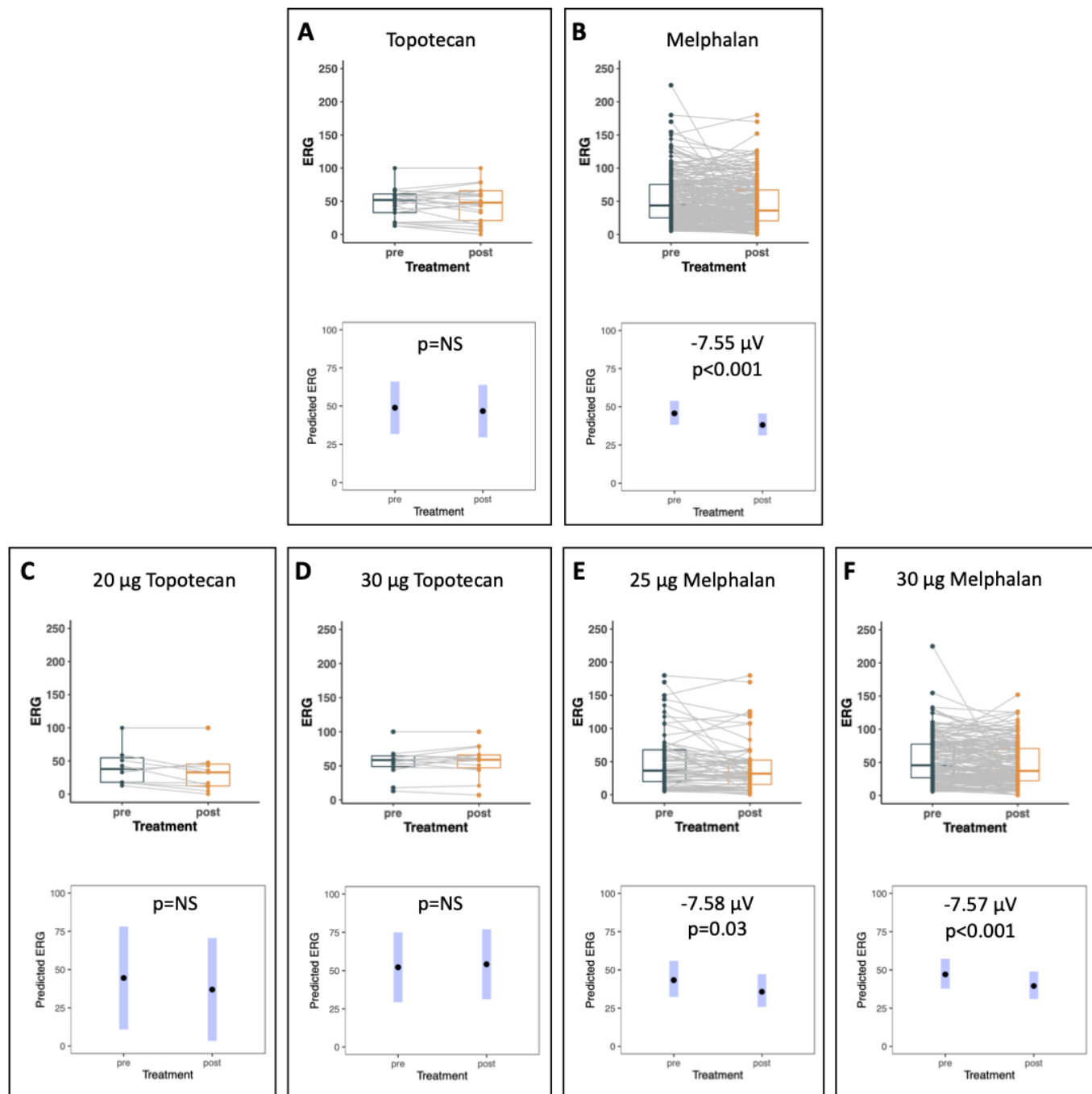
To assess the efficacy, toxicity and optimal therapeutic dose of intravitreal topotecan monotherapy for vitreous seeds, we performed several *in vitro* experiments, *in vivo* experiments in our rabbit model, and we report our clinical experience using intravitreal topotecan in RB patients. Our pharmacokinetic experiments and *in vitro* experiments calculated an optimal dose range of 15–30  $\mu\text{g}$  in rabbits (equivalent to 30–60  $\mu\text{g}$  in the larger human eye). Our *in vivo* efficacy experiments in our rabbit xenograft model demonstrate that 15  $\mu\text{g}$  topotecan is highly effective at eradicating vitreous seeds, with efficacy equivalent to standard dose melphalan. Our *in vivo* toxicity experiments in rabbits demonstrate that multiple injections of topotecan, even up to 30  $\mu\text{g}$ , do not cause retinal functional or structural toxicity, in contrast to melphalan. Finally, in the clinical study, our experience with intravitreal topotecan monotherapy confirms that

30  $\mu\text{g}$  in humans (equivalent to 15  $\mu\text{g}$  in the smaller rabbit eye) does not cause retinal toxicity in patients.

Evidence from animal models<sup>11 12 31 32</sup> and clinical experience,<sup>8 12 33 34</sup> suggests that currently used melphalan may be associated with retinal toxicity. Topotecan has been proposed as an alternative agent with efficacy against RB, and it has been incorporated into chemotherapy regimens by intravenous,<sup>17</sup> intra-arterial<sup>18</sup> and recently intravitreal routes.<sup>19 20</sup> However, topotecan has always been used in combination with other drugs for intravenous<sup>17 35</sup> and intra-arterial regimens.<sup>9 18 36</sup> When topotecan was initially explored for intravitreal use, it was likewise combined with melphalan in an effort to increase efficacy in recalcitrant eyes,<sup>22</sup> but Nadelmann *et al* have shown that combining topotecan with melphalan still causes the expected toxicity from melphalan.<sup>20</sup> Recently, single-agent topotecan has been proposed for vitreous seeds.<sup>19</sup> While some have reported good results, there is much variability in the doses used and many reports still ultimately include melphalan in combination, presumably because of a perceived lack of adequate efficacy at the doses selected for topotecan. A previous evaluation<sup>21</sup> of the toxicity of intravitreal topotecan in an animal model selected a 5  $\mu\text{g}$  dose (equivalent to 10  $\mu\text{g}$  in humans), far less than the doses currently used in clinical practice, six-times less than the dose that we calculate to achieve the  $\text{IC}_{50}$ , and three-times less than the dose we demonstrate to be clinically effective. Importantly, we demonstrate no toxicity with 15  $\mu\text{g}$  or even 30  $\mu\text{g}$  of intravitreal topotecan.

We took an evidence-based approach, rather than an exploratory trial-and-error approach, to determine the ideal dose of topotecan to study. Since each individual injection can only be given at a single location within the globe, while seeds are often diffuse throughout the vitreous cavity, the goal was to identify the concentration required at the farthest-most side of the vitreous to eradicate vitreous seeds at this farthest location. There are different factors to consider, including the rate of diffusion and the rate of efflux. The amount of drug present at the opposite side of the eye at various time-points following injection was therefore determined empirically. *In vitro* cytotoxicity experiments were then performed to determine the minimum concentration necessary at that location based on the pharmacokinetic parameters found *in vivo*, and we then calculated the initial dose that would have been required to be injected to achieve the desired concentration for a sustained length of time (five half-lives) at that farthest point. This systematic approach to dose-finding is superior to selecting several doses in a more random fashion. Since this approach identifies the required concentration at the end of five-half-lives, and at the farthest location in the eye with the lowest exposure levels, this likely represents a high-end estimate—most of the vitreous cavity is exposed to higher concentrations, and indeed at earlier time points the concentration is higher at all locations than it is at the end of the fifth half-life. In addition, this calculated effective dose assumes a single injection, whereas in practice, one would always give multiple injections. Therefore, we also explored half the calculated dose (15  $\mu\text{g}$  instead of the full 30  $\mu\text{g}$ ). Since 15  $\mu\text{g}$  was shown in our rabbit model to be as effective as current melphalan doses, we then explored toxicity at the full 30  $\mu\text{g}$  in our rabbit model as well, and we demonstrate that there is a wide therapeutic window with topotecan.

Similarly, the ERG data of topotecan-treated patients corroborated our rabbit findings that doses of 20  $\mu\text{g}$  or even 30  $\mu\text{g}$  did not cause retinal toxicity or ERG reductions. In contrast, in our mixed effect model, melphalan caused a per-injection reduction in retinal function equivalent to 7.55  $\mu\text{V}$  for every



**Figure 5** Changes in retinal function in topotecan-treated versus melphalan-treated eyes of patients with retinoblastoma vitreous seeds. Within each cohort or subcohort (delineated within a box), the top panel represents the univariate analysis, with each 'string' representing the electroretinography (ERG) changes with a single intravitreal injection. The bottom panel within each pair represents the results of the mixed-effect modelling, accounting for inter-eye/intra-patient and intra-eye correlations, with the appropriate statistical analysis results labelled on the panel. (A–B) ERG amplitude changes per injection for patients treated with topotecan (A), or melphalan (B). Topotecan caused no significant reduction in ERG parameters, whereas significant reductions in ERG amplitudes were seen with each injection of melphalan (7.55  $\mu\text{V}$  per injection,  $p<0.001$ ). (C–F) ERG amplitude changes by drug and dose. (C–D) represent sub-cohorts of the full topotecan-treated cohort presented in (A), and (E–F) represent subcohorts of the full melphalan-treated cohort presented in (B). Topotecan caused no reduction in ERG parameters at either 20  $\mu\text{g}$  (C) or 30  $\mu\text{g}$  (D), whereas significant reductions in ERG amplitudes were seen with each injection of either 25  $\mu\text{g}$  melphalan (E; 7.58  $\mu\text{V}$  per injection,  $p=0.03$ ) or 30  $\mu\text{g}$  melphalan (F; 7.57  $\mu\text{V}$  per injection,  $p<0.001$ ). NS=not significant.

melphalan injection, consistent with previous publications by our group.<sup>12 37 38</sup> There are two commonly used formulations of melphalan: (traditional) melphalan hydrochloride and captisol-stabilised propylene glycol-free melphalan. In the rabbit experiments, all rabbits were treated with traditional melphalan

hydrochloride. In the patient cohort, patients treated up until 2015 (at VUMC) and up until 2016 (at MSKCC) were treated with melphalan hydrochloride, while all patients treated after those dates were treated with the newer propylene glycol-free formulation. We have previously shown that the efficacy and the



toxicity of both formulations do not differ.<sup>38</sup> However, it should be pointed out that not all eyes receiving melphalan will necessarily experience worsened visual function. As seen in figure 5, while there was a reduction in ERG amplitudes *on average*, some eyes experienced little or no ERG reductions. It should also be pointed out that macular toxicity (including cystoid macular oedema), which has been reported to occur occasionally with intravitreal injections,<sup>39</sup> might not result in measurable reduction in retina-wide, full-field ERG. The specific factors influencing retinal toxicity in a given patient are not clear.

## CONCLUSIONS

Taken together, our preclinical and clinical findings support that topotecan 30 µg (equivalent to 15 µg in our rabbit models) appears to cause no retinal toxicity in the rabbit model or in patients. Our rabbit model data indicate that topotecan might be equally effective to melphalan, supporting the need for future clinical studies that directly compare the efficacy in patients.

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