Does a Nanomolecule of Carboplatin Injected Periocularly Help in Attaining Higher Intravitreal Concentrations?

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PURPOSE. To compare intravitreal concentration (VC) of commercially available carboplatin (CAC) and the novel nanomolecule carboplatin (NMC), after periocular injection.

METHODS. The study was a comparative animal study involving 24 white Sprague-Dawley rats, aged between 6 weeks and 3 months. CAC was bound with a nanoparticulate carrier by co-acervation with a biocompatible and biodegradable protein BSA (bovine serum albumin). The particulate size, binding, and structure of the carrier was analyzed with dynamic light-scattering electron microscopy, FTIR (Fourier transform infrared) spectroscopy, and SDS–polyacrylamide gel electrophoresis. Twenty-four white rats were anesthetized. The right eye of each rat was injected with periocular CAC (1 mL) and the left eye with NMC (1 mL) by a trained ophthalmologist. Four mice each were euthanatized at days 1, 2, 3, 5, 7, 14, and 21 and both eyes were enucleated. The intravitreal concentrations of commercial carboplatin and nanomolecule carboplatin were determined with HPLC (high-performance liquid chromatography). Data were analyzed with the paired t-test. The main outcome measure was intravitreal concentrations CAC and NMC over time.

RESULTS. The NMC vitreal concentration was higher than the CAC concentrations in all animals, until day 7 (P = 0.0001). On days 14 and 21, the CAC vitreal concentration was higher than

the NMC concentrations in all animals (P = 0.0002). Overall, the mean vitreal concentration of NMC was greater than CAC.

CONCLUSIONS. Nanoparticulate-bound carboplatin has greater transscleral transport than commercially available carboplatin, especially in the first week after injection and may help enhance the proven adjuvant efficacy of periocular carboplatin over and above systemic chemotherapy in treating human retinoblastoma, especially those with vitreous seeds. This trial is being published to establish a proof of principle for this method of therapy. (Invest Ophthalmol Vis Sci. 2009;50:5896–5900) DOI:10.1167/iovs.09-3914

Retinoblastoma (RB) is the most common primary intraocular malignancy in childhood with an incidence of 1 in 10,000 to 15,000 live births.1–2 Most of the patients are younger than 3 years.2 Patients with bilateral RB have historically been treated with enucleation or external beam radiation therapy (EBRT) or both. Although the efficacy of ionizing radiation therapy has been well-demonstrated, it is now being used less often because of complications such as facial deformities, cataract, and radiation retinopathy and an increased incidence of secondary malignant neoplasms.3 Systemic chemotherapy coupled with appropriate focal therapy has become the current standard of care in the management of RB.4 However, systemic chemotherapy is associated with its own risks. The regimens currently used can cause transient neutropenia, anemia, and thrombocytopenia that may require blood product transfusions and organ toxicities including ototoxicity, renal toxicity, and hepatotoxicity.5 Although the precise risk of nonocular cancers associated with the use of chemotherapy in early childhood in carriers of the RB1 gene mutation remains to be determined, there is reason for concern.6–9 These risks would be minimized if locally administered chemotherapy could replace or at least substitute for some of the systemic chemotherapy. Antineoplastic effects of various agents have been observed after subconjunctival injection for intraocular epithelioma and ocular melanoma in the rabbit, intraocular lymphosarcoma in the cat, and ocular leukemia in humans.6–9 The rationale for using local chemotherapeutic injections is to increase the intraocular concentration of the agents without incurring additional systemic toxicity from increasing intravenous dosages. It has been shown that intravitreal carboplatin is well tolerated in non–tumor-bearing primates and that higher levels of carboplatin are achieved in the vitreous humor when compared with levels achieved after intravenous administration.10 Similarly, it has been shown that intravitreal injections of melphalan in RBs with vitreal seeds achieve higher intravitreal levels, which was important in achieving a success rate of 55.8% of eye preservation in the human eyes so treated.11 Transgenic mice with RB have also shown significant tumor control with subconjunctivally injected carboplatin, accompanied by little or no local toxicity.12,13

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The results of a phase I/II clinical trial of subconjunctival carboplatin in treatment of intraocular RB have shown carboplatin to be effective as well as safe for use. However, another study did not show a beneficial effect in recurrent vitreal or advanced primary RB. Shome et al. evaluated the role of the posterior subtenon (PST) carboplatin injection in addition to intravenous chemotherapy in the only remaining eye of patients with advanced intraocular disease and vitreal seeds. On comparison with a control group of patients with similar disease in whom only intravenous chemotherapy (without PST carboplatin injection) was used, they found extremely high treatment efficacy in the group in which PST carboplatin was used. This trial demonstrated the additional efficacy of PST carboplatin in patients with vitreal seeds. This was thought to be due to transscleral penetration of PST carboplatin leading to augmented vitreal concentrations.

In the light of these results, we hypothesized that reducing the size of the carboplatin molecule may further enhance transscleral osmotic transport through the scleral lamellae and thereby further enhance intravitreal carboplatin concentration. Hence, our group created a novel nanomolecule carboplatin (NMC) and designed an experiment to compare the intravitreal concentration (VC) of commercially available carboplatin (CAC) and the novel molecule of carboplatin coupled with a novel nanoparticulate carrier (NMC), after periocular injection.

Materials and Methods
This was an experimental, comparative, animal study conducted in 24 white Sprague-Dawley rats. The authors confirm adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Biodegradable Carboplatin-Loaded BSA Nanoparticles
Carboplatin was encapsulated with a nanoparticulate form of a protein molecule by co-precipitation and cross-linked by adding an appropriate amount of glutaraldehyde. For the initial trials, BSA (bovine serum albumin) was used, with the possible future use of human (or species specific) serum albumin. Care was taken during this process that adequate glucose be present, as it induces stability in the otherwise labile molecule. The particle size and polydispersity of the BSA nanoparticles was analyzed by photon correlation spectroscopy (PCS) which evaluates the hydrodynamic diameter of the colloidal particle. Simulated lachrymal fluid was prepared and the stability of the nanoparticles was assessed in the fluid over a period of 1 month through dynamic light scattering (DLS). The surface morphology, polydispersity, and particle shape were determined by TEM (scanning electron microscopy). Transmission electron microscopy (TEM) measurement was performed to estimate the stability of the particle size of the formulations. The surface electrical characteristic and its variation with different preparation parameters were determined by ζ-potential measurements. Fourier transform infrared (FTIR) spectroscopy was used to examine the bonding or chemical interaction between carboplatin and the carrier BSA in the lyophilized sample. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was used to analyze and compare the purity of the prepared nanoparticles with pure BSA. HPLC was used to standardize the retention time and the concentration of carboplatin.

Mechanism of the Experiment
Twenty-four white Sprague-Dawley rats were anesthetized with halothane. The right eye of each rat was injected periconjunctivally with CAC. The left eye was injected periocularly with the NMC. Both eyes were injected with 1 mL of the chosen drug in the PST location by an ophthalmologist with specialized training in ocular oncology (DS), experienced in having performed similar injections in patients with RB. Care was taken to avoid penetration of the globe and the globe was evaluated carefully after injection. The animals were then revived and returned to their respective enclosures. Four animals each were euthanized at days 1, 2, 3, 5, 7, 14, and 21 and both eyes were enucleated, by the ophthalmologist (DS). The vitreal concentration of carboplatin, in both eyes, was evaluated with HPLC.

Methodology of Injection Technique
One milliliter of each of the molecules was injected with a 30-gauge needle via a PST route, taking special care to ensure against penetration of the globe. Egress of the carboplatin to the ocular surface was prevented by a cotton-tipped applicator placed at the conjunctival injection site, after needle withdrawal.

Mechanism of Evaluating Vitreal Concentration
After enucleation, the eyes were stored in randomly numbered containers. The technician used a random number generator to store the 48 eyes and maintained the code of the animal, when it was euthanized, and the type of molecule injected around the eye. The complete eye was stored at −80°C in 900 mL of distilled water. The technique of HPLC analysis was standardized. Each eye was then sonicated in ice (for 8 minutes). This step of sonication was included for efficient extraction and release of carboplatin, if adsorbed to membranes and loosely bound to proteins. This step does not cause any redistribution of the drug concentration in the ocular tissue. Vitreous was extracted from the eye and vibrionixed and centrifuged (20 minutes, 8000 rpm). The supernatant was then used for preparation of the ultrafiltrate (1 hour, 8000 rpm). Twenty microliters of this ultrafiltrate was then used for HPLC analysis by the technique used by Zufia et al. and 20 μL was used for determination of protein by the technique used by Lowry and Rosebrough.

Carboplatin concentration by HPLC analysis was investigated using a C18 column (Nucleosil-120-5), an eluent consisting of methanol and an aqueous solution of H2SO4 (0.001 N with Na2SO4 0.02 M; 10:90 or 5:95 vol/vol) and UV detection at wavelength 229 nm.

Statistical Analysis
The intravitreal concentration of CAC and NMC was tabulated. Analysis of data was performed with the paired t test.

Results
The nanoparticles were determined to have spherical shape and size, with a mean diameter of 214 nm, as measured by DLS and confirmed by TEM, although by varying the preparation methodology. The nanoparticles were quite monodisperse with a polydispersity index of less than 0.02 (typically 0.001). The surface morphology was smooth. Vitreal concentration of NMC was much higher (mean concentration, 15.10 μg/mL) in all the animals, in the first week. This difference is statistically significant (P = 0.0001). Conversely, on days 14 and 21, the vitreal concentration of CAC was found to be much higher (mean concentration, 15.10 μg/mL) compared with that of NMC (mean concentration, 1.41 μg/mL). This difference was also statistically significant (P = 0.0002; Fig. 1). The probabilities were significant on each day (1, 3, 5, 7, 14, and 21) for the mean difference in concentration of drugs A and B (details in Tables 1, 2, 3).

All the animals had mild chemosis in both eyes, immediately after the injection; however, it quickly decreased. There was no difference between the two eyes, either in the immediate postinjection phase or in the later stages.
RB management is complex and involves enucleation and other globe-conserving techniques of which chemoreduction is the most popular one.\textsuperscript{18–20} Chemoreduction is most successful for tumors without associated subretinal fluid or tumor-related seeding. Also, chemoreduction can cause systemic side effects and cannot be used if the patient has extraocular invasion by the tumor, systemic metastasis, or inadequate renal, hepatic, or auditory functions. Children with bilateral RB have a strong genetic cancer diathesis and are at a greater risk for mutagenesis and second tumors. This risk has been shown clearly in previous reports when patients with RB were treated with external beam radiotherapy or systemic chemotherapy.\textsuperscript{3,21} The risks can be minimized if these modalities are replaced by chemotherapy delivered locally. We undertook the present study to evaluate whether periocular NMC injections have any therapeutic benefit over periocular CAC injections. We studied 24 rats that received periocular CAC injections in the right eye (1 mL) and periocular NMC injection in the left eye (1 mL). The vitreal concentration of carboplatin, in both eyes, was evaluated with HPLC. The main outcome measure was to analyze and compare intravitreal concentrations of the two drugs over time.

Chemotherapeutic agents used in the present days include vincristine, etoposide, carboplatin, cyclophosphamide, and cyclosporine-A. Cyclophosphamide and vincristine sulfate are among the more effective chemotherapeutic agents, but their use is associated with substantial toxicity that includes bone marrow suppression, nephrotoxicity, and myelotoxicity.\textsuperscript{22} Platinum compounds such as cisplatin and carboplatin, demonstrate relatively low toxicity compared with other chemotherapeutic agents.\textsuperscript{23} Clinical trials\textsuperscript{24} have demonstrated the efficacy of systemic carboplatin therapy in the management of multiple pediatric malignancies (neuroblastoma, Ewing sarcoma, Wills tumor) and adult neoplasias as well. For these reasons, carboplatin is included in the chemotherapeutic regimen for most patients with extraocular as well as intraocular RB. The above have resulted in remarkably effective regression of intraocular RB. As a consequence, such regimens are now used widely as primary chemoreduction therapy, especially in children who have bilateral RB.

Carboplatin is an analogue of cisplatin that causes less toxicity and has been found beneficial in intraocular as well as

<table>
<thead>
<tr>
<th>Drug/Statistic</th>
<th>1 day</th>
<th>3 days</th>
<th>5 days</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (CAC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.04</td>
<td>0.00</td>
<td>1.9</td>
<td>2.77</td>
<td>10.49</td>
<td>19.70</td>
</tr>
<tr>
<td>2 SD</td>
<td>0.00–0.16</td>
<td>0</td>
<td>1.01–3.57</td>
<td>1.49–3.98</td>
<td>8.46–12.12</td>
<td>17.48–21.12</td>
</tr>
<tr>
<td>B (NMC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.89</td>
<td>21.66</td>
<td>15.10</td>
<td>8.01</td>
<td>1.67</td>
<td>1.16</td>
</tr>
<tr>
<td>2 SD</td>
<td>1.83–1.89</td>
<td>18.50–25.14</td>
<td>14.09–16.41</td>
<td>6.23–9.10</td>
<td>1.20–1.97</td>
<td>0.76–1.48</td>
</tr>
</tbody>
</table>

The concentration of group B appeared to be much higher during the early period (up to day 7) and became much lower on days 14 and 21.
extraocular RB.\textsuperscript{25} When administered intravenously, therapeutically level of carboplatin enter the eye and bind firmly to tumor DNA where it forms platinum-DNA adducts. This DNA binding is the mechanism by which the drug kills tumor cells. Carboplatin has been found to be effective against brain tumors and is known to cross the blood–brain barrier.\textsuperscript{26} Despite the relatively favorable toxicity profile for carboplatin, significant side effects have been observed including myelosuppression, nephrotoxicity, ototoxicity, sepsis, second tumors, and death.\textsuperscript{27} Ocular side effects of systemic carboplatin that have been reported include choroiditis, retinitis, maculopathy, optic neuritis, and optic neuropathy.

Harbor et al.\textsuperscript{28} investigated the role of intravitreal injections of carboplatin in transgenic murine RB and found that tumor development was inhibited by intravitreal injections of carboplatin in a dose-dependent manner. They calculated the dose of intravitreal carboplatin resulting in complete tumor cure in 50% of eyes (TCD\textsubscript{50}) in their study to be 1.4 \textmu g. Murray et al.\textsuperscript{12} published the results of their study conducted for evaluating the role of subconjunctival carboplatin therapy with or without cryotherapy in the treatment of transgenic murine RB. They found a dose-dependent inhibition of intraocular tumor growth in their study with TCD\textsubscript{50} being 180 \textmu g. They did not find any histopathologic evidence of ocular toxicity. Hayden et al.\textsuperscript{13} in another murine transgenic RB model reported TCD\textsubscript{50} of subconjunctival carboplatin to be 138.3 \textmu g in 10-week-old mice. Abramson et al.\textsuperscript{29} were the first ones to conduct a phase I/II trial of subconjunctival carboplatin in human intraocular RB. They administered 1.4 to 2 mL of a 10-mg/mL solution of carboplatin subconjunctivally and found good response to the therapy in tumors not associated with subretinal disease. The treatment was well-tolerated by most young children with intraocular RB, with minor local toxicity and no clinically relevant systemic toxicity. This therapy was administered without any concurrent SALT (serial aggressive local therapy) or systemic chemotherapy. However, only 54% of patients in their study group belonged to group Vb, whereas all the patients in a study (100%) by Shome et al.\textsuperscript{15} belonged to group Vb. Ghose et al.\textsuperscript{20} have also reported that subconjunctival carboplatin may have an adjuvant role in therapy for RB. However, their study was also a noncomparative case series and therefore could not substantiate the role of subconjunctival carboplatin. In comparison, the study by Shome et al.\textsuperscript{15} demonstrated the additional efficacy of PST carboplatin in addition to intravenous chemotherapy in comparison with a control group of patients with similar disease in whom only intravenous chemotherapy (without PST carboplatin injection) was used. This study found extremely high treatment efficacy in the group in which adjuvant therapy was used and demonstrated the additional efficacy of PST carboplatin in patients with vitreous seed. The additional efficacy was thought to be due to transscleral penetration of PST carboplatin leading to augmented vitreal concentrations.

In this particular trial, our group prepared a novel molecule of NMC. To the best of our knowledge, this is the first time carboplatin has been made available in a nano form. The stability of the molecule was demonstrated by DLS and TEM and for encapsulation efficiency by HPLC. In this trial, the size-dependent disposition of the nanoparticles after periorcular injection was an important factor. The particle size of the NMC molecule was a mean of 214 nm, as measured by DLS and confirmed by TEM. We plan to evaluate the ocular distribution of this NMC molecule after periorcular injections, as well as the disposition of the NMC in the periocular and ocular tissues, as part of the next phase of the trial.

We found that the vitreal concentration of NMC was much higher (mean concentration, 11.66 \mu g/mL) compared with vitreal concentrations of CAC (mean concentration, 1.17 \mu g/mL) in all the animals, in the first week. This difference was found to be highly significant statistically ($P = 0.0001$). Conversely, on days 14 and 21, the vitreal concentration of CAC was found to be much higher (mean concentration, 15.10 \mu g/mL) than the vitreal concentration of NMC (mean concentration, 1.41 \mu g/mL). This difference was also highly significant statistically ($P = 0.0002$). Our hypothesis for this interesting phenomenon is that the NMC, being a smaller molecule, establishes a stronger osmotic gradient for transscleral migration initially in to the vitreous, compared with CAC. In the later stages, this molecule is probably transported out of the vitreal cavity earlier than the conventional, larger molecule by the vitreal transport mechanisms. Clinically, this early transport may mean that sustained-release devices will be necessary for the distribution of this NMC for maintenance of vitreal concentrations similar to or greater than the conventional molecule.

Among the weaknesses of this trial, the hypothesis of transscleral migration of the NMC would have been further strengthened by performing transport studies using an in vitro transscleral drug exchange model for the NMC and this has been planned. Moreover, the dose of NMC injected was determined on the basis of equivalent carboplatin to be administered by measuring the loading of the drug into the nanoparticles and calculating the equivalent free carboplatin. Even though the same amount of the two drugs was injected periocularly (thereby eliminating the amount of drug injection as a confounding variable), the dose scheduling could have been better demonstrated using the more relevant murine transgenic RB model. This would also have helped evaluate the effect of NMC on tumor burden. The structural stability does not prove clinical efficacy, and hence we are evaluating the NMC in vitro in RB cell lines and plan to use the same in the murine RB model.

This trial proves our hypothesis that NMC has greater transscleral migration (due to a smaller size and greater osmotic diffusion gradient) than CAC. This may in future enhance further the proven adjuvant efficacy of periorcular carboplatin over systemic chemotherapy in treating human RB, especially

### Table 2. Comparison of Two Groups Using Paired $t$-test for the First Week

<table>
<thead>
<tr>
<th>Drug</th>
<th>Observations</th>
<th>Mean</th>
<th>SE</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (CAC)</td>
<td>16</td>
<td>1.17</td>
<td>0.35</td>
<td>0.42 to 1.93</td>
</tr>
<tr>
<td>B (NMC)</td>
<td>16</td>
<td>11.66</td>
<td>1.95</td>
<td>7.50 to 15.95</td>
</tr>
<tr>
<td>Difference</td>
<td>16</td>
<td>-10.49</td>
<td>2.01</td>
<td>-14.79 to -6.19</td>
</tr>
</tbody>
</table>

$t = -5.20$, 15 degrees of freedom. During the first week the concentration of drug B was much higher (mean concentration, 11.66 \mu g/mL) compared with drug A (mean concentration, 1.17 \mu g/mL). The difference is statistically significant ($P = 0.0001$).

### Table 3. Comparison of Two Groups Using Paired $t$-Test for 8–21 Days

<table>
<thead>
<tr>
<th>Drug</th>
<th>Observations</th>
<th>Mean</th>
<th>SE</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (CAC)</td>
<td>8</td>
<td>15.10</td>
<td>1.82</td>
<td>10.78–9.41</td>
</tr>
<tr>
<td>B (NMC)</td>
<td>8</td>
<td>1.41</td>
<td>0.14</td>
<td>1.07–1.75</td>
</tr>
<tr>
<td>Difference</td>
<td>8</td>
<td>13.68</td>
<td>1.93</td>
<td>9.10–18.26</td>
</tr>
</tbody>
</table>

$t = 7.07$, 7 degrees of freedom. On days 14 and 21, the concentration of drug A was much higher (mean concentration, 15.10 \mu g/mL) than that of drug B (mean concentration, 1.41 \mu g/mL). The difference is statistically significant ($P = 0.0002$).
in those with vitreal seeds. This trial is being published to establish a proof of principle for this therapy.

References


