Daunomycin in the Treatment of Experimental Proliferative Vitreoretinopathy

Effective Doses In Vitro and In Vivo

Peter Wiedemann,* Nino Sorgente,† Clara Bekhor,† Randi Patterson,* Tai Tran,* and Stephen J. Ryan*

In previous studies the authors have shown that daunomycin, an anthracycline antibiotic, when injected into the vitreous effectively controls experimental proliferative vitreoretinopathy. Here we show that by administering daunomycin intravitreally it is possible to achieve in vivo concentrations that prevent fibroblast proliferation in vitro. The authors have also determined that the half-life of daunomycin in the vitreous is 131 min, indicating that a critical concentration is maintained in the eye for longer than 4 hr after a single injection. Using 3H-daunomycin, the authors have found that the drug is eliminated across the retina; no significant binding of the drug to vitreous components occurs. These studies demonstrate that it is possible to define the kinetics of drugs injected into the vitreous; and a knowledge of the distribution of any drug in ocular tissues is necessary to effectively determine whether such drug is of therapeutic value. Invest Ophthalmol Vis Sci 26:719–725, 1985

Proliferative vitreoretinopathy (PVR) is the most common complication of retinal reattachment surgery.1 The condition is characterized by the proliferation of cells on both surfaces of the retina, resulting in membrane formation and traction on the retina.2 If the proliferation of cells in the vitreous that results in re-detachment of the retina could be inhibited, more favorable long-term results of surgery would be obtained. Control of cellular proliferation would prevent the secondary processes of contraction and collagen deposition, which lead to traction detachment.

A drug appropriate for the control of PVR must possess two qualities: it has to inhibit cell proliferation effectively, and it must do this without intolerable toxicity to the retina or other ocular structures. The effectiveness depends on the pharmacodynamic and pharmacokinetic characteristics of the drug. The desired pharmacokinetic properties include suitability for local injection into the vitreous, as this results in the highest possible concentration at the target site, thus eliminating or at least reducing systemic toxicity.

The drug must remain in the vitreous long enough to affect the proliferation of cells during a sensitive period of their cycle.

Previous studies have shown the anthracycline antibiotic daunomycin to be effective in the control of experimental PVR. A dose response study in the rabbit showed that the detachment score, which is a measure of the severity of membrane formation and traction detachment following intravitreal injection of cultured fibroblasts, was significantly lower in treated animals than in controls. At high doses, however, there was marked toxicity to the retina, causing detachment and degeneration.

Obviously, it is very important to determine whether it is possible to achieve useful concentrations of the drug at the target site and whether such concentrations last long enough to be of value in treating clinical PVR. To determine what minimal dose has to be maintained for what length of time in the vitreous to prevent proliferation of fibroblasts without toxicity to the retina, we determined cell survival into vitro after exposure to daunomycin and performed pharmacokinetic studies in the rabbit to determine the concentrations of daunomycin and its metabolite daunomycinol in the vitreous and other ocular tissues.

Materials and Methods

Materials

Amphotericin B was purchased from Squibb (Princeton, NJ); Garamycin, from Schering Corpor-
ratiion (Kenilworth, NJ); daunomycin and Sigmacote, from Sigma Chemical Company (St. Louis, MO); \([^{3}H(G)]\)-daunomycin (specific activity, 2.5 Ci/mmol) and Protosol, from New England Nuclear (Boston, MA); fetal bovine serum and RPMI 1640 culture medium, from Irvine Scientific (Santa Ana, CA). Daunomycinol was the generous gift of Dr. Arcamone (Farmitalla; Milano, Italy).

Animals

The investigations utilizing animals, as described in this article, conform to the ARVO Resolution on the Use of Animals in Research. The animals used in this study were maintained in animal care facilities fully accredited by the American Association of Laboratory Animal Sciences.

The Effect of Daunomycin on Colony Forming Units

Rabbit dermal fibroblasts (3rd to 5th passage) were seeded in 35-mm petri dishes (30,000 cells/dish) in RPMI 1640 medium containing 20% fetal bovine serum, 50 μg/ml Garamycin, and 5 μg/ml amphotericin B and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Seventy-two hours after seeding, the cells were treated for 1 hr with various concentrations of daunomycin at final concentrations ranging from 100 to 1000 nM. The fibroblasts were then washed two times with media, trypsinized, and harvested as a single cell suspension. For each dose point, three dishes were pooled, and the suspension was counted in an electronic particle counter (Coulter counter). For each concentration of daunomycin, six petri dishes (60-mm) were seeded at a density of 500 cells/dish. Control cells underwent all manipulations as the treated cells except exposure to drug and received an amount of BSS (vehicle) equal to that of the experimental group.

After 2 weeks, the cells were fixed with methanol for 10 min, and stained with Giemsa, and the number of colonies containing more than 30 cells was determined. The number of cells in a colony was determined using a stereomicroscope. We considered colonies of 30 or more cells to originate from normally reproducing cells. The percentage survival was calculated in reference to the controls, and the logarithm of the percentage was plotted against increasing drug concentration. The experiment was repeated three times.

The extent of cell damage as a function of exposure time was examined by exposing cells to daunomycin (500 nM) for different times between 30 min and 5 hr.

Spectrofluorimetric Assay of Daunomycin

Three sets of six pigmented rabbits of either sex, weighing 2.5 to 3.5 kg, were anesthetized and injected with 10 nmol daumocin into one eye only using a Stepper pipette (Tridak; Highland, NY) and killed after 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, and 6 hr. No paracentesis was performed after the injection. After enucleation, the cornea and lens were removed, and the vitreous was carefully expressed and homogenized on ice. The volume of the vitreous was between 1.3 and 1.5 ml. Daunomycin was extracted from the vitreous by the addition of an equal volume of a 4:1 mixture of 0.1 M phosphoric acid/acetone. The samples were centrifuged for 1 hr at 1000 × g in an IEC Centra 7R centrifuge. The total fluorescence of the supernatants was determined with an Aminco Bowman spectrophotofluorometer (Binney, MA) using an excitation wavelength of 470 nm and an emission wavelength of 585 nm. Daunomycin standards carried through the extraction procedure were used to calculate the amount of daunomycin in the supernatant. The extraction efficiency as determined by mixing 1–6 nmol daunomycin with 1 ml vitreous was 104 ± 6%. Siliconized glassware was used for all procedures.

Light Microscopic Autoradiography

Twenty-five μCi of \(^{3}H\)-daunomycin in a volume of 100 μl (= 10 nmol daunomycin) were injected into one eye of four rabbits to determine whether there was loss of drug across the retina after intravitreal injection. Two rabbits were killed after 1 hr; the remaining two rabbits were killed after 2 hr. A corneal button was removed, and the eyes were fixed by immersion in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for several days and then processed for glycol methacrylate embedding. Three-micrometer sections were prepared on a Reichert/Jung Model 1140 Autocut (AO) (Chatsworth, CA), and sections were mounted on acid-cleaned glass slides. Under safelite conditions, slides were dipped in NTB-2 Nuclear Track emulsion (Kodak; Rochester, NY), mixed 1:1 with distilled water, and heated to 42°C, dried for 2 hr and stored in a light-tight box with drierite (Ted Pella; Tustin, CA) at 4°C. After 1 week, the slides were developed for 3 min in D-19 developer (Kodak) at 20°C with constant agitation, rinsed briefly, and then fixed in Rapid Fix (Kodak) for 3 min. After rinsing for several
minutes, the sections were stained with 2% Richardson's stain, dried, coverslipped, and viewed with the Zeiss photomicroscope.

Scintillation Counting of Radioactive Daunomycin in Ocular Tissues

Daunomycin and 3H-daunomycin were mixed, and 100 μl of a BSS solution containing 100 nmol/ml and approximately 2 nCi/ml daunomycin was injected into one eye of four rabbits. The animals were killed after 1 and 2 hr, respectively. The eyes were enucleated, kept on ice, and processed immediately. Adherent episclera was cleaned off. The aqueous was removed with a tuberculin syringe and a 27-gauge needle, then the cornea was excised at the limbus, the lens removed, and the vitreous carefully expressed. The eye was opened, and retina and choroid together were scraped from the sclera with a scalpel blade. Each tissue was weighed and placed in a scintillation vial. Protosol (2 ml) was added to each vial, and the vials were incubated at 55°C for 24 hr to solubilize the tissues. H2O2 (200 μl) and acetic acid (50 μl) were added to suppress quenching and chemoluminescence. Scintiverse II (Fisher Scientific; Tustin, CA) (10 ml) was added. A control eye, injected with BSS only, was processed in the same way as the experimental eye. The samples were counted in a Beckman LS9000 liquid scintillation counter (Beckman; Fullerton, CA).

For the evaluation of the data, the mean counts of the two experimental animals for each time point were corrected for tissue weight (to a weight that was calculated as the mean weight of the respective tissue of all 5 animals), and the blank value (untreated eye) was subtracted.

Vitreous and Lenticular Binding of Daunomycin

In siliconized vials, dilutions of daunomycin were prepared at a final concentration of 1, 4, 7, 10, 15, 20, 25, 50, 100, 200, 250, 500, and 1000 nmol/ml if 100 μl was added to 2 ml homogenized vitreous. To this mixture of vitreous and daunomycin we added 2 nCi tritiated daunomycin. After mixing with a Vortex mixer the vitreous was placed in a Spectrapor dialysis membrane (2000 molecular weight cut off) (VWR) and placed into 50 ml cold BSS; it was dialyzed and stirred in the cold. After 20 hr, the radioactivity of the vitreous in the dialysis bag and of 1 ml of the dialysate was determined. The experiment was repeated four times, each time in triplicate.

To determine whether the lens-bound daunomycin, freshly removed rabbit lenses were stirred for 24 hr in 4.5 ml ice-cold BSS containing 10 nCi 3H-daunomycin and unlabelled daunomycin at final concentrations of 1 μM, 10 μM, and 50 μM daunomycin. After 24 hr, radioactivity was determined in 1 ml of supernatant. All determinations were done on triplicate samples.

High Performance Liquid Chromatography (HPLC)

We adapted a method for the quantitative measurement of daunomycin in the vitreous, using a Beckman 344 HPLC system equipped with an Altex ultrasphere C8 column and a UV detector (Beckman model 165). The column was developed with acetonitrile/H3PO4 0.01 M (50:50) and 0.005 M hexane sulfonic acid. The flow rate was 1.5 ml/min and the absorption wavelength was 500 nm. The sample size applied to the column was 20 μl.

Results

Colony Formation Assay

Killing of asynchronous cells as a function of increasing daunomycin concentration was shown by an exponential decrease of the number of colony
forming units (CFU) after the cells were exposed to concentrations of daunomycin varying between 100 and 1000 nM (Fig. 1). The concentration of daunomycin that caused a 50% inhibition of CFU after 1 hr exposure was 700 nM. Prolongation of exposure time to 5 hr resulted in complete inhibition of fibroblast reproduction at a daunomycin concentration of 500 nM (Fig. 2). Staining of cells with trypan blue is often used as a criterion of cell death. By this criterion, immediately after the cells were exposed to any daunomycin concentration for 1 hr, cell viability was 70–95%.

**Half-Life of Daunomycin in the Vitreous**

As shown in Figure 3, the drug was cleared from the vitreous following a first order kinetic with a half-life of approximately 2 hr. The highest measured concentration at 30 min after injection of 10 nmol was 4.4 µM. When 10 nmol daunomycin were injected into an enucleated eye, concentrations between 7.3 and 8.5 µM were measured; this was in agreement with values obtained when the drug was added to expressed vitreous in vitro, where we measured a concentration of 7.5 µM, suggesting that the collection of vitreous was complete.

**Autoradiography**

Autoradiography showed drug in all retinal layers around the posterior pole (Fig. 4). The peripheral retina contained fewer grains; radioactive daunomycin was found in the ciliary body and a small number of grains was evident in the lens and sclera. In the area of the retina that contained most of the grains, we observed unspecific retinal damage, such as slight disruption of inner and outer nuclear layers, and shortening and thickening of the photoreceptor outer segments; the photoreceptor outer segments appear to be the most sensitive structure in the eye with regard to daunomycin toxicity.

**Daunomycin in Ocular Tissues and Vitreous and Lenticular Binding**

Direct measurement of radioactivity in ocular tissues (Table 1) after the injection of 10 nmol daunomycin demonstrated that most of the drug was in either the vitreous or retina; practically none was found in the aqueous and cornea. When lenses were incubated in vitro with daunomycin, we could not demonstrate any loss of radioactivity from the incubation medium indicating that very likely the lens does not bind daunomycin. Likewise, we were not
able to demonstrate any binding of daunomycin to the vitreous.

**High-Performance Liquid Chromatography**

When a 10 μM standard of daunomycin was applied to the column, only one peak was obtained, showing that the drug is pure. During elution of the peak, a wavelength scan (200–600 nm) was performed. The curve obtained was the same as one obtained by scanning a daunomycin solution with a Beckman DU7 spectrophotometer. The same peak was obtained when daunomycin was mixed with vitreous in vitro before chromatography or when a sample of rabbit vitreous that had been injected with 100 nmol daunomycin after enucleation was chromatographed. An eye injected in vivo and enucleated after 1 hr or 4 hr showed two peaks. The second peak, whose height increased with time, was identified as daunomycinol by comparing its elution time with that of a daunomycinol standard, determining the absorbance ratio \( \lambda_1/\lambda_2 \) (\( \lambda_1 = 254 \text{ nm} \); \( \lambda_2 = 233 \text{ nm} \)), possible detection at 500 nm (anthracycline absorbance maximum at this wavelength), and absorbance scanning during elution, where we found the same absorbance maxima as for the standards (Fig. 5).

**Table 1. Determination of radioactivity (dpm) in various ocular tissues 1 and 2 hr after injection with Daunomycin (10 nmol; 7200 dpm)**

<table>
<thead>
<tr>
<th>Time after injection</th>
<th>1 hr</th>
<th>2 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cornea</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Lens</td>
<td>140</td>
<td>258</td>
</tr>
<tr>
<td>Vitreous</td>
<td>2666</td>
<td>1367</td>
</tr>
<tr>
<td>Retina/veal</td>
<td>3046</td>
<td>4059</td>
</tr>
<tr>
<td>Sclera</td>
<td>746</td>
<td>479</td>
</tr>
<tr>
<td>Total</td>
<td>6598</td>
<td>6163</td>
</tr>
</tbody>
</table>

* Data corrected for weight of tissue.
Discussion

Selection of the proper daunomycin concentrations and exposure times in vitro and in vivo is a complex task. Essentially the effectiveness of a drug depends on the concentration of the drug at the target site, the time during which an effective concentration is present at the target site, as well as on the kinetics of the cell population at the time it is exposed to the drug. With regard to the effective concentration, probably the time of exposure to superthreshold concentrations is more important than peak concentrations.

Cell death is a multistage process and manifests itself in morphologic degeneration, metabolic death, or reproductive death. The inability to reproduce should be the only relevant criterion to assess cell kill after use of an antiproliferative drug, since metabolic death does not represent a valid measure of the viable cell population. The goal of this study was to quantitatively determine loss of reproductive integrity in vitro and correlate this with drug concentrations and exposure times achievable in vivo. Our results with fibroblasts, in agreement with the literature, show a logarithmic decrease of CFUs as a function of increasing daunomycin concentration. The efficacy of different agents on a single cell type and the activity of one drug on different cell classes can be determined and compared by using the colony formation test.

This is important, as different cell lines may show different sensitivity to various drugs. We showed the inability of fibroblasts to proliferate, i.e., form colonies, when they were exposed to 1 μM daunomycin for 1 hr or 500 nM for 5 hr.

Depending on the vitreous volume (1–1.5 ml), and assuming an even distribution of daunomycin in the vitreous, a dose of 10 nmol, the therapeutic dose in experimental PVR in the rabbit, will result in a maximal concentration of 6.6–10 μM daunomycin. Even though there is loss of drug from the injection site, our results show that a dose that is effective in vitro can be achieved and maintained in vivo for at least 4 hr. The close correlation between the inhibitory concentrations obtained in vitro and in vivo indicate that the colony formation assay can be used to predict effective drug concentrations in vivo, thus sparing the life of experimental animals.

We have shown that there is no significant binding of daunomycin to the vitreous or the lens. We also were able to show that the drug is metabolized to less effective daunomycinol, but from our data it is not possible to determine the site of metabolism. It seems generally accepted that the diffusion of solutes in the vitreous, even if they have a large molecular weight, appears to be unrestricted. We were therefore surprised when we found the drug concentrated around the optic disc in the autoradiographs, whereas the peripheral retina was essentially free of drug. From Figure 4 it is obvious that there is more drug in the inner and outer nuclear layer than in the photoreceptor outer segment layer. As the 1- and 2-hr autoradiographs and the determination of radioactive drug in the ocular tissues (Table 1) show only the immediate deposition of daunomycin and not its final place of action, we did not further quantitate the distribution of daunomycin using grain counts.

We have previously noted that a dose of 9 nmol/eye daunomycin resulted in alteration of the photoreceptor outer segments. However, no other clinical and histologic signs of toxicity were evident. Since there was a time period of 28 days between the injection of drug and enucleation in that study, the damage to the retina observed here may only be transient due to retinal edema. If more extensive studies on the toxicity of low doses of daunomycin will show that the structural changes we have noted are not permanent, daunomycin may be useful as a therapeutic drug for PVR.

Key words: colony formation assay, proliferative vitreoretinopathy, rabbit, autoradiography, scintillation counting, spectrofluorometry, daunomycin
Acknowledgment

The authors thank E. Cogan, PhD, of Beckman Instruments for his advice and for the generous use of an HPLC.

References