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Research Paper

Human Scleral Diffusion of Anticancer Drugs from Solution and Nanoparticle Formulation

Esther S. Kim,[1,5,6] Chandrasekar Durairaj,[2] Rajendra S. Kadam,[2] Sung Jin Lee,[1,3] Yun Mo,[4] Dayle H. Geroski,[1] Uday B. Kompella,[2] and Henry F. Edelhauser[1]

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Purpose. To determine the transscleral permeability of chemotherapeutic drugs vinblastine and doxorubicin for treatment of intraocular tumors, and to compare the use of doxorubicin encapsulated in PLGA and liposome nanoparticles.
Methods. Human sclera was isolated and mounted in a Lucite chamber. Fluorescently tagged vinblastine (VIN), innately fluorescent free doxorubicin (DOX), PLGA doxorubicin (PLGA-DOX), or Doxil (Tibotec Therapeutics) were added to the episcleral donor chamber. The choroidal side was perfused with Balanced Salt Solution. Perfusate fractions were collected over 24 h and measured for fluorescence. Following the experiment, tissue sections were imaged, underwent a drug wash out procedure, and tissue drug content was analyzed using an LC–MS/MS method.
Results. Within 24 h, a total of 68%, 74%, 29%, and 1.9% of the drug dose from VIN, DOX, PLGA-DOX, and Doxil, respectively, diffused across the sclera. VIN and DOX scleral tissue showed strong fluorescence after 24 h. PLGA-DOX displayed scattered fluorescence, and Doxil indicated minimal fluorescence. LC–MS/MS revealed strong tissue binding of DOX.
Conclusions. This study suggests both vinblastine and doxorubicin are able to diffuse across human sclera. In addition, PLGA nanoparticles delivered doxorubicin at a slower rate across the sclera, and the liposome preparation resulted in the slowest delivery of drug.

KEY WORDS: chemotherapeutic; liposomes; nanoparticles; retinoblastoma; transscleral.

INTRODUCTION

Chemotherapy is an important part of treatment for retinoblastoma, an intraocular tumor. Current regimens for systemic chemotherapy include varying combinations of vincristine, carboplatin, etoposide, and adjunct cyclosporine (1–4). Despite their promising results, these treatments are not without their drawbacks. Systemic chemotherapy has been associated with myelosuppression, febrile episodes, subsequent infections, need for blood transfusions, vincristine neurotoxicity, organ toxicities, and dehydration (2,5–7).

Many researchers have investigated more localized treatment of the eye for retinoblastomas. Van Quill *et al.* (8) and Murray *et al.* (9) found inhibition of intraocular tumor growth in murine models with subconjunctival injections of carboplatin in fibrin sealant, and using carboplatin with and without cryotherapy, respectively. Another group has found that carboplatin is able to penetrate the human sclera *in vitro* and rabbit sclera *in vivo* (10). These encouraging results have led to a phase I/II study in children, which has found subconjunctival carboplatin as an effective treatment for intraocular retinoblastoma (6). The purpose of this study is to begin investigating the usefulness of localized transscleral delivery to the eye for doxorubicin and vinblastine, two anticancer agents that are of value in treating retinoblastoma (11,12). The current study evaluated the *in vitro* transscleral diffusion of vinblastine (VIN) and doxorubicin (DOX) using human sclera. In addition, the transscleral delivery of doxorubicin from poly(lactide-co-glycolide) (PLGA) nanoparticles (PLGA-DOX) and Doxil liposomes was assessed.

VIN is frequently used to treat Hodgkin's disease, several malignant lymphomas, Kaposi's sarcoma, and testicular cancer (13). VIN is also an analog of vincristine, which is currently used systemically for retinoblastoma treatment. VIN's mechanism of action involves capping microtubule ends, thus inhibiting microtubule assembly within the tumor cells (14).

DOX has been successful in treating neoplastic conditions such as leukemia, neuroblastomas, soft tissue and bone sarcomas, breast cancer, bladder cancer, thyroid cancer,

[1] Department of Ophthalmology, Emory University School of Medicine, Atlanta, Georgia, USA.
[2] Department of Pharmaceutical Sciences and Ophthalmology, University of Colorado, Denver, Colorado, USA.
[3] Department of Ophthalmology, College of Medicine, Soonchunhyang University, Seoul, South Korea.
[4] Forest Research Insitute, Commack, New York, USA.
[5] 1365B Clifton Rd. NE, Room B2515, Atlanta, Georgia 30322, USA.
[6] To whom correspondence should be addressed. (e-mail: esther.soon@gmail.com)

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Hodgkin's disease, and malignant lymphomas. DOX's mechanism of action involves binding to DNA, and inhibiting topoisomerase II to prevent replication within the tumor cells (13). However, DOX has been associated with toxicity, and a total amount accumulated should not exceed 550 mg/m^2 (7).

PLGA-DOX is a more novel drug form of doxorubicin encapsulated in poly (lactide-co-glycolide) polymer nanoparticles. Previous studies indicated that doxorubicin loaded poly-β-hydroxybutyrate microspheres injected intravitreally can result in slow release of the drug in ocular tissues (15). Thus, the sustained release of doxorubicin from PLGA nanoparticles could offer sustained release at a lower concentration transsclerally and provide a less invasive and less toxic delivery of doxorubicin for intraocular tumors.

Doxil (Tibotec Therapeutics) is doxorubicin encapsulated in STEALTH liposomes for intravenous use. This formulation was selected because of its current clinical availability and to use it as a direct comparison to PLGA-DOX nanoparticles. It is currently used to treat HIV-related Kaposi's sarcoma and ovarian cancer that has progressed or recurred after prior platinum based therapy (Doxil [package insert], Tibotec Therapeutics, Raritan, NJ).

MATERIALS AND METHODS

Drug Preparation

Vinblastine (MW: 810.974) was fluorescently tagged with BODIPY FL to yield MW: 1,043.02 with maximum excitation/emission at 503/512 nm (Invitrogen). The BODIPY FL dye is relatively nonpolar and electrically neutral. To reconstitute 100 μg of vinblastine (MW: 1,043.02), it was dissolved in 50 μL DMSO and 950 μL BSS to produce a 0.1 mg/mL solution.

Doxorubicin hydrochloride (Sigma) is an innately fluorescent molecule with an Excitation/Emission of 470/585. 1 mg of doxorubicin (MW: 579.98) was reconstituted using 200 μL DMSO and 850 μL BSS to form a 0.488 mg/mL solution.

Preparation and Characterization of Doxorubicin Loaded Nanoparticles

Doxorubicin hydrochloride loaded poly (lactide-co-glycolide) (PLGA) nanoparticles were prepared by an emulsion/solvent evaporation method in the Kompella laboratory. Briefly, doxorubicin hydrochloride (1 mg) was dissolved in 1 mL of deionized water. This solution was added to 1 mL of 20 mg/mL human serum albumin (HSA) and kept shaking at 600 rpm at room temperature for 4 h. The incubated solution was dialyzed against 1 L of deionized water (MWCO 1,000) for 12 h to remove the unbound free drug. The complex was lyophilized and the doxorubicin–HSA complex was dissolved in 1 mL of borate buffer (pH 8.3). This solution was added to 4 mL of dichloromethane containing 100 mg PLGA 85:15 (inherent viscosity 0.63 dL/g) and sonicated at 9 W for 1 min. The emulsion formed was added to 25 mL of 2% PVA solution and sonicated at 39 W for 3 min. The final emulsion formed was stirred for 1 h followed by rotary evaporation for 3 h. The emulsion was centrifuged at 34,155×*g* for 30 min and the pellet obtained was washed twice with 10 mL of deionized water and finally redispersed in 10 mL of deionized water and lyophilized. The drug content was 6.71 μg of doxorubicin/mg nanoparticle. The effective diameter of the nanoparticles was 265.5±4.8 nm. The nanoparticles disperse readily in aqueous solution, and a 0.488 mg/mL concentration of DOX in nanoparticles was used for each experiment.

Doxil is a clinically available from Tibotec Therapeutics and was purchased from the Emory University Pharmacy. These pegylated Doxil liposomes are approximately 100 nm in size and are available in solution at 2 mg/mL. The 2 mg/mL concentration was used in our experiments. This concentration is 4 fold higher than the DOX and PLGA-DOX used.

In Vitro Release Profile of Doxorubicin from PLGA Nanoparticles

To determine the rate of doxorubicin drug release from the PLGA nanoparticles, a release profile was performed. Weighed amount of nanoparticles (~10 mg in triplicates) was dispersed in 0.5 mL of PBS (pH 7.4). The dispersion was placed in a dialysis bag of MWCO 10,000 (Spectra/Por 7 dialysis tubing, Spectrum Laboratories Inc., CA) and both the ends were sealed with dialysis clips. The sealed dialysis bags were placed in 25 mL of PBS (pH 7.4) and kept for incubation at 37°C with 150 rpm (Barnstead, IL). For comparison, 0.5 mL of doxorubicin aqueous solution (0.5 mg/mL) was taken in a dialysis bag and kept for *in vitro* release in PBS. At regular time intervals 1 mL of sample was taken and replaced with 1 mL of fresh PBS. The withdrawn samples were analyzed for fluorescence by spectrofluorometer (excitation wavelength = 470 nm; emission wavelength = 580 nm).

Experiment Setup

Human donor scleral poles were obtained from the Georgia Eye Bank. The mean donor age at time of death (±SD) was 60±9.3 years (range 43–72). The tissue was stored in moist chambers at 4°C and used 1.7±0.94 days post-enucleation. A study by Olsen *et al.* found no significant difference in hydration between 3 h BSS perfused sclera, and the 2-day-old and 4-day-old moist chamber stored globes, which supports tissue viability (16).

To obtain bare sclera, the intraocular tissues were removed from the poles, and the sclera was cleaned using cellulose surgical spears (Ultracell). While removing the extraocular tissues, including conjunctiva and extraocular muscles, the tissue was kept moist by placing it briefly in Balanced Salt Solution (BSS). After cleaning, the scleral tissue was then cut and mounted horizontally with episclera up, in a Lucite block perfusion chamber as published in Rudnick *et al.* (17) and Gilbert *et al.* (18). The sclera was placed between two elastomer rings (Sylgard) 8.0 mm in diameter to prevent edge damage and lateral leakage. The exposed scleral surface area was ~50 mm^2. The perfusion chambers were maintained at 37°C with a circulating water bath.

The upper, or donor chamber, holds the drug. The lower, or receiving chamber, holds a volume of approximately 500 μL and was perfused continuously with BSS using a pump (Harvard Apparatus). The solution in the receiving

chamber was kept homogenous using a micro magnetic stir bar, and outflow was connected to microtubing raised 22 cm above the sclera to achieve 15 mmHg pressure. The drug that diffused across the sclera combined with BSS and was collected into test tubes using a fraction collector, which rotated to a fresh tube every 2 h.

BSS was allowed to perfuse for several minutes at 1 mL/min through the receiving chamber before beginning the experiment to ensure no leakage and to remove all bubbles. Once no leaks were found, the flow rate was decreased to 3 μL/min. The experimental drug (200 μL) was added to the episcleral surface. Because the sclera has been found to have inherent fluorescence, adjacent sections of scleral tissue from the same donor eye were mounted in a separate perfusion chamber, and 200 μL of BSS was added as a control experiment. Perfusate fractions (~360 μL) were collected every 2 h for 24 h for VIN, DOX, PLGA-DOX, Doxil, and corresponding BSS controls. The fluorescence in each fraction was measured using a TD-700 fluorometer (Turnerbiosystems), which was calibrated using 5–10 known concentrations of drug, ranging from 12.5 to 0.4 μg/mL of vinblastine, and 48 to 0 μg/mL of doxorubicin. Apparent transscleral permeability, K_{trans}, was calculated at the peak steady state as:

$$K_{trans} = \frac{R_{total}}{A \times t} \times \frac{1}{[D]}$$

Where R_{total} represents the total moles of drug that diffused through the sclera in time *t*. *t* is defined as the duration of steady-state flux. *A* is the surface area of the sclera (square centimeter) across which the flux is measured. *D* is the original concentration of the drug in the donor chamber (moles per milliliter).

Immediately following the 24 h experiment, the sclera was removed from the chamber. The exposed portion of sclera was blotted dry with filter paper, cut with a 7.5 mm trephine, and then cut in half and each part weighed. Half of the sclera was frozen and mounted for unstained cryosections, while the other half was frozen to use for a scleral wash out procedure the following day.

Wash Out Procedure

The frozen half of human scleral tissue was thawed, and placed into a well of a 24 well tissue culture plate containing 1 mL BSS. The tissue was allowed to extract in the well for 30 min at room temperature. It was then transferred to a fresh well of BSS. This process was repeated for a total of 6 h. After the wash out, samples were taken from each well, and fluorescence was measured to determine the amount of drug that diffused out of the sclera. To normalize the results, drug concentration was divided by the weight of the tissue used for the wash out.

Imaging

After experimentation, scleral tissue was mounted with O.C.T. Compound (Tissue-Tek) and cut on a cryostat for unstained cryosections. Images of the tissue were taken using an epifluorescent microscope and a cooled digital camera. To ensure comparable images, we applied the same settings when capturing drug exposed and control images.

LC–MS/MS Analysis of Doxorubicin and Vinblastine

Doxorubicin and vinblastine accumulation in albino rabbit scleral tissue were estimated by LC–MS/MS analysis. Briefly the tissue samples were homogenized in 500 μL of deionized water by tissue tearor (Biospec Product, Racine, WI, USA). To this tissue homogenate 50 μL of 1 μg/mL of budesonide was added as internal standard and mixed thoroughly by vortexing at medium speed for 5 min on multi tube vortexer. Ethyl acetate (2.5 mL) was added to this mixture and vortexed for 15 min at medium speed. The organic layer was separated by centrifugation at 3,000×*g* for 10 min. The separated organic layer was pipette out in clean glass tube and evaporated under nitrogen stream (N-evap Organomation, Berlin, MA, USA). The residue after evaporation was reconstituted in 500 μL of acetonitrile, centrifuged at 5,000×*g* for 5 min, and 250 μL of supernatant was transferred to the LC–MS vials for analysis. An API-3000 triple quadrupole mass spectrometry (Applied Biosystems, Foster City, CA, USA) coupled with a Perkin Elmer liquid chromatography system was used. Analytes were separated on Hypersil-ODS C18 column (2.1×100 mm, 3.9 μm, from Thermo-Electron Corp. Waltham, MA) in isocratic elution mode using acetonitrile and aqueous buffer (80:20 *v*/*v*) at flow rate of 0.3 mL/min. The buffer was 5 mM NH_4COOH in water pH adjusted to 3.5 with formic acid. Doxorubicin, vinblastine and budesonide (internal standard) molecular species were analyzed in positive mode of ionization with declustering potential and collision energy optimized for each analyte. Multiple reactions monitoring for two analyte and internal standard were selected with dwell time of 0.2 s. The following transitions were monitored: 544/397 (doxorubicin); 811.4/355 (vinblastine); 431/413 (budesonide). The retention time for doxorubicin, vinblastine and budesonide were 3.7, 2.8 and 2.5 min respectively.

RESULTS

Vinblastine (VIN)

Fig. 1 shows the cumulative flux amount of vinblastine and BSS measured using a fluorometer (n=5). Over a 24 h period, approximately 68% was able to diffuse across the human scleral tissue (Table I). No significant fluorescence was found in the BSS control samples in comparison. Peak diffusion of 1.83 μg occurred at 10 h. Using the peak steady-state diffusion period of vinblastine from hours 6–16 (Fig. 2A), the apparent K_{trans} was calculated as $4.98 \pm 0.56 \times 10^{-6}$ cm/s (Table I).

Fig. 2 shows sclera exposed to vinblastine for 24 h and the corresponding control tissue. After 24 h, a strong fluorescent green vinblastine is visible in the sclera. The choroidal side is in contact with a flow of BSS, which may explain the absence of complete saturation on this side of the sclera. The BSS control sclera (Fig. 2B) shows minimal fluorescence in comparison to exposed tissue.

The VIN wash out studies (n=5) following a 24 h experiment indicate that most of the vinblastine diffused out

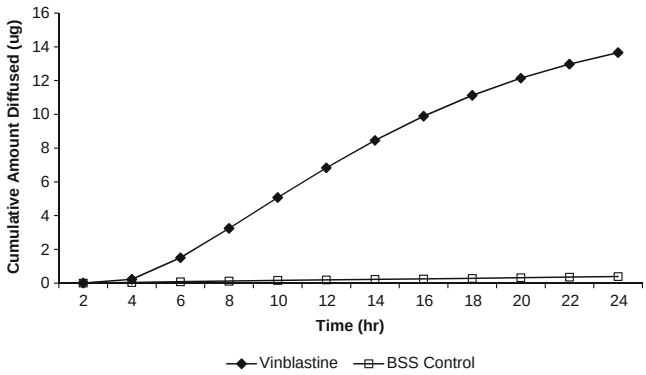

Fig. 1. Cumulative amount of transscleral vinblastine diffusion over 24 h and control (n=6).

of the sclera within 3 h, and $t_{1/2}$ occurred at approximately 45 min. Approximately 5.3 μg vinblastine was released over 6 h from vinblastine exposed sclera (Table I). Using LC–MS/MS, mean tissue drug content ±SD was measured for n=6 and determined to be 1.13±0.39 ng/mg tissue.

Doxorubicin (DOX)

Fig. 3A and B show the hourly and cumulative amount of doxorubicin that diffused across the sclera (n=8). Peak diffusion of 8.15 μg occurred at 8 h, and approximately 74% of DOX perfused across the sclera over 24 h (Table I). Using a peak steady-state diffusion period of doxorubicin from hours 6 to 14, apparent K_{trans} was calculated as $3.50\pm0.31\times10^{-6}$ cm/s (Table I).

After 24 h of exposure to DOX, the sclera becomes completely saturated with the yellow-orange fluorescence of the DOX. DOX remains in the scleral tissue after a 6 h wash out period (n=4), and $t_{1/2}$ occurred between 3.0 and 3.5 h. Approximately 50 μg doxorubicin washes out of the exposed tissue over a 6 h period, and mean scleral drug content ±SD (n=5) was measured following 24 h exposure to DOX using LC–MS/MS and determined to be 212±69 ng/mg tissue (Table I).

In Vitro Release Profile of PLGA-DOX Using a Dialysis Bag (MWCO: 10,000)

Doxorubicin hydrochloride had a linear release in PBS from 0.0488 to 3.125 μg/mL. Doxorubicin solution was completely released in 10 h (100%) as shown in Fig. 4. However, the drug release from PLGA nanoparticles was less than 0.0488 μg/mL till 6 h. At 24 h, only 3.39% of drug was released from the nanoparticles. The cumulative release of doxorubicin at 168 h (7 days) was 12.72±1.89% from the nanoparticles. These results clearly indicated sustained release of doxorubicin from the nanoparticles with nearly no burst-effect and the potential of this delivery system to release doxorubicin at a controlled rate over months.

PLGA Doxorubicin (PLGA-DOX)

The cumulative diffusion of PLGA doxorubicin (n=5) across human sclera is shown in Fig. 3B. Nanoparticles are not expected to permeate significantly across the sclera. We previously observed that 200 and 2000 nm particles are almost completely retained in the periocular space for 2 months after posterior subconjunctival injection in a rat model (19). These nanoparticles were not detectable in the rat intraocular tissues for up to 2 months. In addition, we previously determined that 200 nm particles do not permeate across bovine sclera in 24 h, while 20 nm particles permeate only to the extent of 0.46% (20). Since we used 265 nm PLGA particles, PLGA-DOX diffusion was calculated using free doxorubicin (DOX: Ex/Em: 470/585) standards to determine the amount of DOX that diffused across the sclera. Peak diffusion of 3.62 μg occurred at 10 h, with a steady decrease in diffusion after this time point. During a 24 h experiment, 27% of the original doxorubicin was able to cross the sclera (Table I). Apparent K_{trans} of PLGA-DOX was calculated using hours 8 to 14 and determined to be $2.53\pm0.22\times10^{-6}$ cm/s (Table I).

The epifluorescent images of the sclera exposed to doxorubicin nanoparticles displayed orange dots of doxorubicin fluorescence scattered within the sclera after 24 h exposure to the lamina fusca surface.

During washout, the PLGA-DOX (n=4) showed a gradual diffusion of doxorubicin from the sclera over 6 h. $t_{1/2}$ occurred between 3.0 and 3.5 h. A total of 4.8 μg PLGA doxorubicin washed out of scleral tissue over a 6 h period. LC–MS/MS analysis of scleral drug content ±SD (n=4) was determined to be 25.7±3.7 ng/mg tissue (Table I).

Doxil

Transscleral flux of Doxil (n=4) is shown in Fig. 3A. As with the PLGA-DOX, free doxorubicin (DOX) was used as standards to determine the amount of free DOX that diffused. Peak diffusion of 1.00 μg occurred at 8 h, with a tapering off in flux after this time point. A total of 1.9% of doxorubicin was able to cross the sclera during a 24 h experiment. Apparent K_{trans} of Doxil was calculated using hours 8 to 12 and determined to be $4.74\pm0.73\times10^{-7}$ cm/s (Table I).

Table I. Summary of Transscleral Diffusion Over a 24 h Period, as Observed in this Study

Drug	Donor (μg)	Diffused in 24 h (μg)	Apparent K_{trans} ±SE (cm/s)	Wash out in 6 h (μg)	Scleral drug content LC–MS/MS (ng/mg tissue)
Vinblastine	20	13.7=68.3%	$4.98\pm0.56\times10^{-6}$	5.3	1.13±0.39
Doxorubicin	97.6	72.6=74.4%	$3.50\pm0.31\times10^{-6}$	50	212±69
PLGA doxorubicin	97.6	27.9=28.6%	$2.53\pm0.22\times10^{-6}$	4.8	25.7±3.7
Doxil	400	7.65=1.9%	$4.74\pm0.73\times10^{-7}$	1.5	3.22±1.2

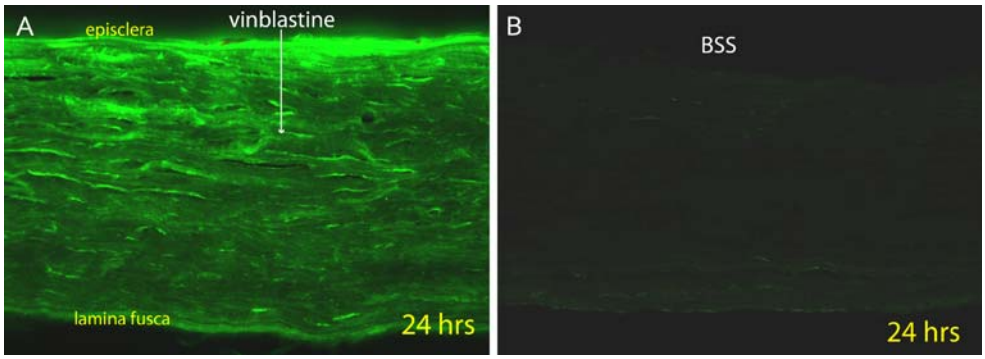

Fig. 2. Epifluorescent images of scleral tissue exposed to **A** vinblastine for 24 h and **B** corresponding BSS control tissue. (×100 magnification).

Epifluorescent images of the sclera exposed to Doxil indicate minimal yellow-orange doxorubicin fluorescence on the episcleral surface only and none within the sclera.

Doxorubicin release from the Doxil exposed sclera for 6 h following experimentation (n=4) showed a $t_{1/2}$ of approximately 5 h. Minimal drug was recovered from the sclera with a total of 1.5 μg doxorubicin recovered over 6 h, and scleral drug content ±SD (n=5) was measured as 3.22± 1.2 ng/mg tissue using LC–MS/MS.

DISCUSSION

The results of these studies have determined that vinblastine and doxorubicin are able to diffuse across the human sclera in a 24 h period. BODIPY-vinblastine had 68% diffusion of the original drug over 24 h while free doxorubicin attained 74% diffusion (Table I). Molecular size is not the sole determinant of a drug's transscleral permeability. A study by Ambati *et al.* has found that globular proteins diffuse more readily through rabbit sclera than linear proteins of similar molecular weight (21). Varying physicochemical factors such as radius, charge, and solubility of the drug can affect each drug's ability to permeate the sclera. Although fluorescein (MW: 332) is almost a third the molecular weight of BODIPY-vinblastine conjugate(MW: 1,043), it had a similar transscleral permeability rate of $5.21\pm0.71\times10^{-6}$ cm/s (22) compared to vinblastine at $4.98\pm0.56\times10^{-6}$ cm/s. Vinblastine alone has a molecular weight of 811 Da compared to the BODIPY-vinblastine conjugate used in the experiments with a MW: 1,043 Da. This difference in molecular weight could affect the diffusion of vinblastine, but as these permeability rates show, smaller size may not always increase diffusion rate. Similarly, doxorubicin (MW: 580) is able to permeate the sclera faster than a slightly smaller molecule, rhodamine (MW: 479) at a rate of $3.50\pm0.31\times10^{-6}$ cm/s *vs.* $1.86\pm0.39\times10^{-6}$ cm/s, respectively (22).

Results from the doxorubicin containing drugs suggest that free doxorubicin binds to scleral tissue to a greater extent than nanoparticle encapsulated formulations. Fluorescence microscopy displayed PLGA-DOX as scattered dots of fluorescence within the sclera in contrast to the complete saturation of fluorescence with DOX exposed tissue; these results suggest more homogenous scleral binding with free doxorubicin. The wash out studies and LC–MS/MS also indicate much higher levels of DOX retained in the sclera when compared to nanoparticles. After 6 h of wash out, a

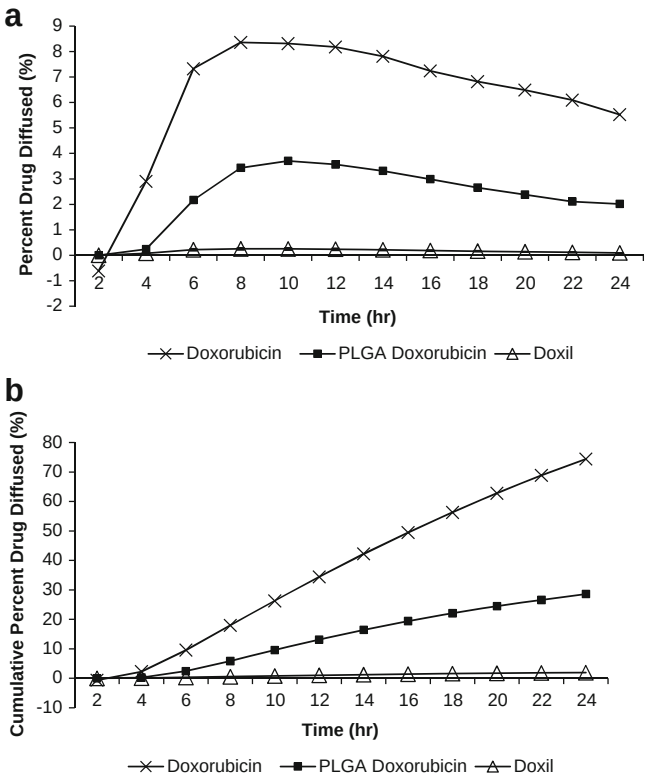

Fig. 3. A Comparison of percent transscleral diffusion (±SE) of doxorubicin-containing drugs over 24 h, and **B** cumulative percent of drug diffused.

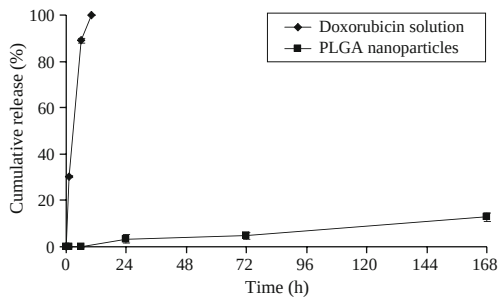

Fig. 4. Cumulative release of doxorubicin in PBS buffer from solution and PLGA nanoparticles.

total of 50, 4.8, and 1.5 μg of doxorubicin were recovered from DOX, PLGA-DOX, and Doxil, respectively (Table I). LC–MS/MS analysis of scleral drug content following 24 h experimentation showed 212, 25.7, and 3.22 ng/mg tissue in the DOX, PLGA-DOX, and Doxil exposed tissues, respectively.

Fluorescent images of Doxil correspond to its low permeability with minimal fluorescence on the episcleral surface, and no fluorescent signal elsewhere within the tissue. Less than 2% of the original doxorubicin released from Doxil was able to diffuse across the sclera over the 24 h period (Table I). The minimal wash out of drug from Doxil tissue (1.5 μg) is consistent with the minimal diffusion of Doxil across the sclera and/or release of free DOX from its liposomes, and also scleral drug content measurements using LC–MS/MS. This data suggests that a liposome placed on the episclera is not able to permeate the hydrophilic sclera. However, because of the Doxil slow release, there is the potential for a decrease in toxic effects as well. There is a possibility that the Doxil liposomes might be beneficial in clinical setting for treating retinoblastoma, due to their slow release properties. However, it is unclear if the liposomes would retain their integrity in the biological surroundings of the eye for long durations.

When comparing free DOX with PLGA-DOX, we observe that PLGA-DOX has a lower amount of doxorubicin diffusion at 29% compared to DOX diffusion at 74% (Fig. 3B). The low rate of doxorubicin release from nanoparticles is consistent with the sustained release profile of doxorubicin from PLGA nanoparticles. The *in vitro* release profile predicted release of 12.7% over 7 days using a dialysis bag of MWCO: 10,000. One possible explanation for the greater release of drug across sclera compared to the *in vitro* release study is that the drug may have migrated to the surface of particles between the time of preparation and the time of use in transport studies.

Greater sustained release with nanoparticles and microspheres has been documented in previous animal studies, such as the study by Kompella *et al.* that found upon subconjunctival injection of budesonide nanoparticles into rats, nanoparticles were able to provide sustained release of budesonide for up to 7 days, which plain budesonide did not (23). Between microparticles and nanoparticles, microparticles sustained budesonide release for more prolonged periods, possibly due to the lower burst release of drug from microparticles. A study by Tao Hu *et al.* (15) also found lower doxorubicin levels in rabbit ocular tissue following intravitreal injection of doxorubicin loaded poly-β-hydroxybutyrate microspheres than when compared to free doxorubicin. The current *in vitro* study shows that nanoparticles can provide a degree of sustained delivery. With PLGA-DOX, beyond the rapid initial burst release observed in the *in vitro* permeability study, the remaining drug content is likely to be released at a slower rate, consistent with slow degradation of the polymer. For cytotoxic drugs such as doxorubicin, slow release over a week or a few days is likely more appropriate in order to minimize side effects while achieving clinical outcomes.

In summary, the current studies have shown that both vinblastine and doxorubicin can diffuse across the human sclera as well as becoming a depot within the sclera. We also determined that PLGA-DOX and Doxil preparations can slow the release and permeability of doxorubicin across human sclera. These findings support the studies by Abramson *et al.* (6) and Van Quill *et al.* (8) that periocular chemotherapy for retinoblastoma for chemoreduction is feasible. In patients with stage IV retinoblastoma, chemoreduction with systemic vincristine, cisplatin, etoposide, and doxorubicin have been effective. However, there are indications where a transscleral approach may be more effective to reduce toxicity because the chemotherapeutic agents can be placed on the sclera directly adjacent to the tumor tissues within the eye. Such localized application of drugs enhances drug delivery to the target retina by several fold, allowing dose-reduction compared to the systemic route (24). Such transscleral approach of anticancer drug delivery might be of special value for a high risk population of patients with optic nerve involvement and/or orbital involvement (11).

Past research on the delivery of other transscleral drugs (triamcinolone) has shown that the conjunctival blood vessels and lymphatics can easily remove drugs that have been injected subconjunctivally and/or sub-tenons (25). In addition, prior studies have shown that 200 nm particles in periocular space are not subject to such rapid clearance by blood and lymphatic vessels (19,20). Thus, a delivery device and/or sustained vehicle such as nanoparticles may be needed to extend the time of the vinblastine and/or doxorubicin adjacent to the sclera and other orbital tissues. Further elucidation of the pharmacokinetics of nanoparticles *in vivo* is necessary. This study demonstrated the suitability of transscleral approach in delivering anticancer drugs at various rates, with the use of slow release delivery systems.

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