The Effect of Ocular Pigmentation on Transscleral Delivery of Triamcinolone Acetonide

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Abstract

Purpose: To determine the capacity and kinetics of the binding between triamcinolone acetonide (TA) and the ocular pigment for a better understanding of the transscleral delivery.

Methods: In the *in vitro* study, natural melanin (*sepia officinalis*, Sigma-Aldrich) was incubated at 37°C with different concentrations of TA and the binding capacity/binding affinity was measured. The TA releasing profile from the melanin was also studied through repeated incubation of TA-melanin in fresh phosphate-buffered saline. In the *ex vivo* study, the effect of the choroidal pigment on the trans sclera/choroid permeability of TA was investigated through Franz-type vertical diffusion cells using both a TA suspension and a saturated TA solution.

Results: The amount of TA bound to melanin increases with the increase of the TA concentration and with an increase in the incubation time. A Scatchard analysis revealed that the maximum number of moles of TA bound to melanin is predicted to be 22.43 nmol/mg, with a binding affinity of $K = 2.4 \times 10^{-5} \text{ nM}^{-1}$. TA released from a pigment showed a fast phase within the first 24 h and a slow phase thereafter. About 40% of the bound TA released in the first day and 73.94% of accumulative release was observed after 5 days. The TA suspension showed more TA penetration through the scleral-choroid complex than the saturated solution ($P = 0.0104$). The apparent permeability coefficients for the suspension across the sclera-choroid of pigmented and albino rabbits are $7.48 \pm 1.53 \times 10^{-6} \text{ cm/s}$ and $10.78 \pm 2.49 \times 10^{-6} \text{ cm/s}$, respectively.

Conclusions: TA can bind to and release from the ocular pigment, which may extend the TA ocular half-life and therapeutic duration when TA is delivered through a subtenon injection. A further *in vivo* study is warranted to validate the findings and to quantitate the magnitude of the difference between pigmented and albino animals.

Introduction

TRIAMCINOLONE ACETONIDE (TA) is widely used in clinical practice for the treatment of posterior ocular diseases such as diabetic macular edema,^{1,2} age-related macular degeneration,³ and uveitis.⁴ TA can be administered either through an intravitreal injection or through a subtenon injection. The route of the intravitreal injection is more invasive and is associated with a higher rate of complications such as vitreous infection, vitreous hemorrhage, cataract, and elevation of intraocular pressure.^{5,6} In contrast, the subtenon injection or transscleral delivery has many advantages. The human sclera has a large surface area (16–17 cm²) accounting for 95% of the total globe surface,⁷ which provides an effective place for the interaction between the drug and the eye globe. In addition, the sclera is a much more durable tissue compared with the retina. The studies also demonstrated that the sclera has good permeability for many

types of drugs, including small molecules,⁷ peptides,⁸ and even proteins.⁹

TA is a small molecule compound and its good lipophilicity and moderate water solubility¹⁰ may render it particularly suitable for transscleral drug delivery into the eye. We and others have demonstrated that a single subtenon TA injection can provide a long therapeutic drug level in the choroid, retina, and vitreous.^{11–15} We found that the TA concentration in the choroid was 152 times higher than that in the vitreous following a single subtenon injection of 40 mg.¹⁴ We hypothesized that TA might have been bound to the ocular pigment in the choroid. A recent publication by Thakur et al. showed that 6 corticosteroids, including TA, all have the ability to bind to natural melanin.¹⁰ However, quantitative binding such as the binding capacity and affinity between TA and the ocular pigment have not yet been reported in the literature. The current study was designed to quantitate the

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binding capacity and affinity as well as characterize the binding kinetics.

Methods

In vitro studies of the binding and releasing kinetics

TA-melanin binding. One milligram of natural melanin (*sepia officinalis*, Sigma-Aldrich) was placed in a glass tube and 2 mL of a standard TA (Toronto Research Chemicals, Inc.) solution at the concentration of 100 ng/mL, or 316 ng/mL, or 1 µg/mL, or 10 µg/mL was added into the tubes, 3 tubes ($n=3$) for each concentration at each sampling point. The tubes were kept under 37°C in a thermostatic shaker at the speed of 100 rpm (481 Forma Orbital Shaker; Thermo Electron Corporation). After 1 hour, 3 hours, 8 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, and 5 days the tubes were centrifuged at 1258g for 10 min (Eppendorf 5810R) and a 200 µL supernatant was collected from each tube for TA quantitation. The amount of TA bound to the pigment was derived by subtracting the TA in the supernatants from the known amount of TA in the initial TA solution used. The maximum moles of the TA bound and the affinity constants were calculated from a Langmuir-like adsorption isotherm.

Release of TA from melanin. One mg of pigment was weighted into a conical glass tube and 2 mL of the TA concentrations (0.1 µg/mL, 0.32 µg/mL, 1 µg/mL, and 10 µg/mL) was added for binding ($n=3$ for each concentration, 12 tubes total). Three tubes were used for each TA concentration. After 24 h of binding at 37°C in a thermostatic shaker at the speed of 100 rpm, the tubes were centrifuged at 1258g for 10 min and the supernatant was removed for TA analysis. The amount of bound TA was taken as the difference between the amount initially added and the amount remaining in the supernatant. At each 24 h, the tubes were centrifuged and the supernatant was replaced with 500 µL PBS. The procedure was repeated and the supernatant was sampled for 5 days. The amount of TA in the supernatant was determined by high-performance liquid chromatography (HPLC). The accumulative TA release was plotted versus the sampling time points, stratified by TA concentrations tested.

Ex vivo studies of TA and pigment binding during transscleral TA delivery

Isolation and preparation of eye wall. Four New Zealand White rabbits ($n=4$) and 4 Chinchilla pigmented rabbits ($n=4$) were used. Their average body weight was 2.50 ± 0.5 kg. Animals were handled in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. The rabbits were sacrificed and the eyes were immediately enucleated. The eye globe was divided into anterior and posterior hemispheres by a circumferential cut 2 mm behind the limbus. The vitreous was removed using a 2-mL syringe without the needle attached. The posterior eye cup was cut into 2 halves through the optic nerve along the medullary ray. A 12-mm diameter disc was taken from the inferior half of the eye cup using a trephine and the center of the circular sample was about 8 mm away from the lower edge of the optic nerve head. The retina and the retinal pigment epithelium (RPE) layer were carefully scraped off with a fine spatula. The remaining 12-mm diameter disk of the Bruch's

membrane-choroid-sclera complex was used for the TA diffusion study. We have noted that it is inevitable that the RPE will more or less come with the retina when the retina is detached from the complex of RPE-Bruch's membrane-choroid.^{14,16} In contrast, one can get a very clean Bruch's membrane-choroid-sclera complex by completely scraping the single layer RPE off from Bruch's membrane under an ophthalmic surgical microscope. To use the Bruch's membrane-choroid-sclera complex without the RPE layer in this study meant to reduce the variation from the possible variable amount of RPE remaining on the Bruch's membrane.

Transscleral diffusion of soluble TA. The Franz-type vertical diffusion cell was used for the experiments. The diffusion cell consists of a donor chamber and a receptor chamber. The disc of the sclera-choroid complex was placed between the 2 chambers. The sclera side faced the donor chamber and the choroid side faced the receptor chamber. The commercial TA (Kenalog-40; Bristol Myers Squibb Princeton) vial was centrifuged for 5 min at 1258g. The 0.6 mL of supernatant was aspirated and pooled from 3 commercial vials. Unlike the *in vitro* studies, which used standard TA to determine the binding capacity and kinetics, the *ex vivo* studies used commercially available preserved TA (Kenalog-40) to simulate a clinical situation in which preserved TAs are commonly used for subtenon injections.^{2,4,11,13,17} The TA concentration of the pooled supernatants was determined by HPLC to be 14.17 ± 0.4 µg/mL. A volume of 0.25 mL of the pooled supernatants was loaded into the donor chamber of the diffusion cell. The receptor chamber was filled with 4.5 mL balanced salt solution (BSS) and caution was paid to make sure there was no air between the disc of the scleral-choroid complex and the BSS. Both outlets of the donor chamber and receptor chamber were sealed with parafilm. The chambers' temperature was maintained at 37°C by the commercial circulating system. At the predetermined time points (10 min, 30 min, 1 h, 3 h, 8 h, 12 h, and 24 h) 100 µL of BSS was taken from the receptor chamber for the TA quantitation and 100 µL of fresh BSS was added back immediately to restore the original volume.

Transscleral diffusion of TA suspension. In this study, the setting of the animal scleral-choroid disc and the diffusion cell were the same as the soluble TA diffusion test. Instead of loading the TA supernatant, 0.25 mL of the TA suspension (20 mg/mL) was loaded. After the last sampling at 24 h, the tested scleral-choroid complex was unmounted and the choroid was carefully harvested for TA quantitation. The permeability coefficient of sclera-choroid for TA was calculated from kinetics of TA in the receptor chamber, according to the expression of Fick's law on passive diffusion:

$$P_a = \frac{V}{A} C_d^{-1} \left(\frac{dC_r}{dt} \right)$$

In this equation, dC_r/dt represents the slope of the linear regression of the cumulative amount of the solute transported over time; A is the area of the tissue surface (19.625 mm^2) available for transport; and C_d is the concentration of the dissolved drug in the donor compartment.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation. The amount of TA bound to the pigment was associated with the

FIG. 1. Time course of triamcinolone acetate (TA) accumulation on the pigment stratified by the escalating TA concentration.

incubation time and TA concentration using a linear regression model. The accumulative amount of TA released from the pigment was compared among the binding TA concentrations using the one-way ANOVA *post hoc* test. Mean TA concentrations in the receiving chambers between pigmented and albino rabbits as well as between the TA suspension and saturated TA solution were paired at each sampling time point and compared using a paired *t*-test. The TA quantity in the choroid between the pigmented and albino rabbits was compared using the *t*-test. All statistical analysis was performed using JMP version 10 and $P < 0.05$ was considered significant.

Results

Kinetics of binding of TA to pigment in vitro

The kinetics of TA binding to pigment is depicted in Fig. 1 and Fig. 2. TA binding to pigment was expressed as a function of incubation time (Fig. 1) and a function of drug concentration (Fig. 2). The binding had a fast phase within the first hour and a gradual increase phase within the rest of the study period (total 5 days). The amount of TA bound to melanin increased with an increase of TA concentration ($P < 0.0001$) and with an increase in incubation time ($P < 0.0001$). A Scatchard analysis was performed on data

FIG. 2. Nonlinear regression fitting of the TA-pigment-binding curve and Scatchard plot (inset).

derived from 5-day incubations using GraphPad Prism (GraphPad Software, Inc.). The maximum number of moles of TA bound to melanin was predicted to be 22.43 nmol/mg, and the binding affinity $K = 2.4 \times 10^{-5} \text{ nM}^{-1}$ (Table 1, Fig. 2).

Release of TA from pigment in vitro

In this study, a repeated sampling strategy was used to obtain accumulative release. There was about 40% of the bound TA released within the first day, and further release was observed with a subsequent incubation in fresh BSS (Fig. 3), except for the release from the lowest TA concentration used for binding. The release reached a plateau between 3 and 5 days. The accumulative release at 5 days was 73.94% from the highest TA concentration used for binding (10 µg/mL) (Table 2).

Binding of TA to biological pigment in rabbit eye

Both a soluble TA trans-scleral-choroid diffusion test and a suspension TA trans-sclera-choroid diffusion test demonstrated that TA penetration into the receptor chamber increased over time. The penetration or TA concentration in the receptor chamber was lower with pigmented rabbits than that with albino rabbits in both the TA supernatant and suspension studies within 24 h of the experiment ($P = 0.035$, 1 tail). Compared to the TA supernatant, TA suspension showed more TA penetration through the scleral-choroid

TABLE 1. MELANIN-BINDING AFFINITY AND CAPACITY OF TA AND THE OTHER COMPOUNDS REPORTED

Compound	Medium/pH	Temperature °C	Affinity (k) (nM^{-1})	Capacity (nMoles/mg melanin)	Reference and citation#
TA	PBS/7.4	37	2.4×10^{-5}	22.43	Current study
Chloroquine	PBS/7.4	37	4.26×10^{-4}	1.13×10^3	Ono C and Tanaka M. #24
Ciprofloxacin	PBS/7.4	37	4.32×10^{-4}	359	Ono C and Tanaka M. #24
Celecoxib	PBS/7.4	?	7×10^{-5}	383	Cheruvu NP, et al. #25
Timolol	*PBS/7.4 (PBS/7.4)	37 (37)	$*6 \times 10^{-3}$ (2.22×10^{-5})	*6.98 (233)	Kadam RS, et al. #27 (Ono C and Tanaka M.) #24
Atropine	PBS/7.4	37	2×10^{-5}	7.6	Shimada K, et al. #26

*Determined from a cocktail solution of β -blockers, including timolol, with melanin.
TA, triamcinolone acetate; ?, not specified.

Graph Builder

FIG. 3. Time course of TA releasing from the pigment for various TA-binding solutions.

complex ($P=0.0104$, Fig. 4). The apparent permeability coefficients for the suspension of TA across the sclera-choroid of pigmented and albino rabbits are $7.48 \pm 1.53 \times 10^{-6}$ cm/s and $10.78 \pm 2.49 \times 10^{-6}$ cm/s, respectively. The amount of TA retained in the choroid during the 24-h experiment was significantly higher in pigmented rabbits than that in the albino rabbits ($P=0.03$, 1 tail, Fig. 5).

Discussion

The accumulation and retention of drug residues in the pigmented ocular tissues is widely acknowledged.^{18,19} Some drug ocular toxicity have been related to its binding to the ocular pigment,²⁰ while other drugs, such as atropine, have been used to its advantage to bind to the ocular pigment.²¹ The binding of a drug to the ocular pigment affects the drug ocular pharmacokinetics and pharmacodynamics.²² Therefore, ocular drug delivery calls for the study of qualitative and quantitative aspects of the drug-melanin interaction, which is particularly relevant to trans-scleral drug delivery because of abundant pigments in the choroid and RPE.

In the current study, we demonstrated that TA did bind to the pigment. The amount of TA bound to the pigment is positively associated with TA concentrations. The binding may be described as an infinitely weak binding in which the TA affinity to the pigment-binding site is influenced by TA

osmolality or concentration. The highest TA concentration used in this study was $10 \mu\text{g/mL}$, which is close to the solubility of Kenalog-40 ($12.5 \pm 0.44 \mu\text{g/mL}$) in PBS at a pH of 7.44 and a temperature of 37°C measured at our laboratory. From the perspective of clinical TA application, the concentration of TA in the choroid following a subtenon TA injection would be comparable to the concentration under which TA binds to the ocular pigment. Under this TA concentration, $9.7 \mu\text{g}$ of TA could be bound to each mg of the ocular pigment if sufficient interaction time is provided. TA binding to the pigment can be expressed as a function of the incubation time. Although the binding increased with an increase of incubation time, a significant amount of binding (about 50% of the observed total binding) was completed within the first hour of the incubation.

The association of many drugs with melanin has been reported²³ and many of these bindings are a reversible

TABLE 2. PERCENTAGE OF TA RELEASED FROM THE MELANIN

Binding TA solution	Percent of TA released (1 day)	Percent of TA released (5 days)	All Pairs Tukey-Kramer 0.05*
0.1 $\mu\text{g/mL}$	11.22	13.39	C
0.32 $\mu\text{g/mL}$	35.69	51.84	B
1 $\mu\text{g/mL}$	43.62	54.48	A B
10 $\mu\text{g/mL}$	42	73.94	A

*Levels not connected by the same letter are significantly different.

FIG. 4. Time course of TA penetration from the donor chamber into the receptor chamber for the saturated TA solution and TA suspension.

FIG. 5. Amount of TA retained in the choroid of albino rabbit (1, AR) and pigmented rabbits (2, PR) after 24 h of permeation of TA suspension through the sclera. TA was retained more significantly in the pigmented choroid.

process.²⁴ As shown in the current study, the TA binding to the pigment was also reversible. Once the surrounding TA concentration was lower, TA could be released from the bound state with half of the bound TA released into the surrounding media within 24 h. In general, a higher binding affinity will lead to a faster drug–pigment association and a longer residence time for the drug at its pigment-binding site. In the current study, the TA-pigment-binding affinity was $K=2.4 \times 10^{-5} \text{ nM}^{-1}$, which was similar to the binding affinity reported for celecoxib,²⁵ timolol,²⁴ and atropine,²⁶ but roughly 18-fold lower than chloroquine and ciprofloxacin (Table 1).²⁴ The reversible binding process is advantageous for treatment of choroidal diseases using TA. The ocular pigment in the choroid can accumulate a high concentration of TA and extend its therapeutic duration accordingly, as it is a well-known fact that atropine provides a much longer mydriatic effect than tropicamide due to atropine's binding to the ocular pigment. It is also possible that the same subtenon 20 or 40 mg of TA injection may provide a longer therapeutic duration in a more pigmented eye. It was reported that the lipophilic drug, peloxacin, had twice as long a vitreous half-life in the pigmented rabbit as compared with that in the albino rabbit.²²

TA binding to the pigment was also observed in our *ex vivo* study in which twice as much TA was retained in the choroid of the pigmented rabbit eyes as compared to the albino rabbit eyes and less TA reached the receptor chamber through the pigmented scleral–choroid complex. A similar observation was also reported with celecoxib.²⁵ The binding capacity of the pigment for different drugs is different due to the available numbers of binding sites or the pigment–drug interaction sites on the surface of the pigment. In the current study, the TA-pigment-binding capacity was 22.43 nanomoles per milligram of melanin (Table 1). This is similar to the reported atropine pigment-binding capacity (7.6 nanomoles per milligram of melanin),²⁶ but lower than the binding capacity for the other drugs reported (Table 1).^{24,25} From those reported drug–melanin-binding capacities,^{24,27} chloroquine²⁴ has the highest pigment-binding capacity (1.13 μMoles per mg of melanin). Other than TA, dexamethasone is also a common corticosteroid used for sub-

conjunctival or subtenon injections.^{28,29} It has been reported that dexamethasone has a higher melanin-binding affinity than triamcinolone¹⁰ although dexamethasone has a much shorter ocular half-life than TA.^{15,29} In addition, it is interesting to note in our study that more TA was transported into the receiving chamber when using the TA suspension instead of the saturated TA solution in the donor chamber regardless to the pigment or albino sclera–choroidal complex used. This suggests that direct contact between a TA crystal and sclera may have a more efficient trans-scleral movement than a real TA solution does. This phenomenon warrants further investigation. In summary, TA can bind to the ocular pigment, which can function as a storage site for TA. The reversible nature of the binding and release may benefit the treatment for choroidal diseases when TA is delivered through the subtenon injection. An *in vivo* study using both pigment and nonpigment eyes will shed more light on the hypothesized difference as well as the magnitude of the difference.

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Author Disclosure Statement

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