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Research Paper

Binding of Betaxolol, Metoprolol and Oligonucleotides to Synthetic and Bovine Ocular Melanin, and Prediction of Drug Binding to Melanin in Human Choroid-Retinal Pigment Epithelium

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Purpose. To characterize the binding of betaxolol, metoprolol and oligonucleotides to synthetic and bovine ocular melanin, and to predict the binding to melanin in human choroid-retinal pigment epithelium (RPE).
Materials and Methods. The shape, size and specific surface area of synthetic melanin and isolated melanin granules from bovine choroid-retinal pigment epithelium (RPE) were characterized by SEM, laser diffractometry and BET. The binding of betaxolol, metoprolol, fluorescein isothiocyanate (FITC)-labeled phosphodiesther oligonucleotides and 6-carboxyfluorescein (6-CF) to melanin was determined. The binding of beta-blockers to melanin in human choroid-RPE was estimated based on binding parameters and the melanin content in human choroid-RPE.
Results. Bovine melanin granules were round or oval with a mean diameter of ca. 1 μm. Synthetic granules were slightly smaller and irregular and had a two times higher specific surface area than bovine melanin. Synthetic melanin bound more betaxolol and metoprolol than bovine melanin and both melanin types showed a high affinity and a low affinity binding sites. The human choroid-RPE was predicted to contain 3–19 times more melanin bound drug than unbound drug at typical therapeutic concentrations (1–1,000 ng/ml). FITC-labeled oligonucleotides and 6-CF did not bind to melanin.
Conclusions. The binding of lipophilic drugs to biological melanin differs from that of synthetic melanin. Lipophilic beta-blockers are expected to bind significantly to melanin in human choroid-RPE: only a small fraction of the drug being in active free form. In contrast, phosphodiesther oligonucleotides do not seem to bind to melanin.

KEY WORDS: beta-blocker; binding; choroid; melanin; RPE.

INTRODUCTION

Melanins are polyanionic biological pigments (1,2). Natural melanin is packed in spherical or ellipsoid melanin granules, also referred as melanosomes or pigment granules. In a granule, melanin is attached to a protein matrix and this melanoprotein complex is surrounded by a thin membrane (3). In ocular tissues, melanin granules are found in the uvea and the retinal pigment epithelium (RPE). Ocular melanin absorbs visible light and protects the retina from overexposure by preventing scattering of light in the eye. In addition to ocular tissues, melanin is found in inner ear, skin, hair, hair follicles and brain (substantia nigra and locus coerelius; 4).

In drug therapy ocular melanin requires consideration since melanin binds free radicals and chemicals, such as drugs, by electrostatic forces and possibly also by van der Waals forces or charge transfer (5). In most cases the binding is reversible. Melanin binds many classes of drugs, e.g. antidepressants, antibiotics, anaesthetics and beta-blockers (5,6). Based on literature, Leblanc *et al.* (6) concluded that all basic and lipophilic drugs can reasonably be expected to bind to melanin. Melanin binding has several pharmacological consequences. Much attention has been given to a possible link between melanin binding, drug accumulation and retinal toxicity, (e.g. chloroquine and phenothiazines; 6). Melanin binding in the iris-ciliary body affects the short-term and long-term drug concentrations in these tissues for example in the case of atropine and timolol (7,8). Melanin binding in the iris-ciliary body may also affect drug response: larger doses are needed to obtain a response, since the melanin bound drug is not available for receptor binding (9). In addition, the melanin bound drug may form a depot that releases the drug over a long time, thereby

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ABBREVIATIONS: BET, Brunauer, Emmet, Teller-method; CF, carboxyfluorescein; DHIC, dihydroxyindole-2-carboxylic acid; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N*-′2-ethane sulphonic acid; HPLC, high performance liquid chromatography; PB8, potassium phosphate buffer, pH 8; RPE, retinal pigment epithelium; SEM, scanning electron microscopy; TFA, trifluoroacetic acid; VEGF, vascular endothelial growth factor.

prolonging drug action (10). In choroid-RPE melanin may affect the permeation of drugs into the retina and vitreous after transscleral drug delivery (e.g., after subconjunctival administration) or during systemic exposure. We have found earlier that the permeation lag time of lipophilic beta-blockers through bovine choroid-RPE in vitro is much longer that that of hydrophilic beta-blockers and this may be due to the melanin binding of lipophilic compounds (11).

The affinity of drugs to melanin is determined by using in vitro binding studies (12,13). In these experiments the interaction between a drug and melanin may be characterized by determining the capacity and affinity of the binding process (13). These in vitro parameters may be correlated with the in vivo binding results or they can be used in pharmacokinetic simulation models. Synthetic melanin is widely used in binding studies, although it's chemical composition and morphology are different from biological melanin (1,14). There are few comparative studies in which the binding parameters of drugs have been determined for synthetic and biological melanin. Potts found that polycyclic compounds bound both to biological and synthetic melanin (12). Recently Koeberle *et al*. (15) determined the binding parameters for one drug candidate, memantine, using synthetic, sepia and bovine ocular melanin and they found differences between the melanin types. However, the pharmacokinetic consequences of the binding of drugs to melanin in choroid-RPE have not been estimated.

To further characterize the differences of synthetic and isolated melanin, we studied the physical characteristics of synthetic and bovine ocular melanin and the binding of two positively charged beta-blockers (betaxolol and metoprolol) to these melanin types. The binding of negatively charged oligonucleotides and 6-carboxyfluorescein to bovine melanin was assessed. The binding of beta-blockers to melanin in human choroid-RPE was estimated at therapeutically relevant concentrations using the binding parameters obtained with bovine ocular melanin.

MATERIALS AND METHODS

Isolation of Melanin Granules from Bovine Eye

The experiments were accomplished according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fresh bovine eyes were obtained from a local abattoir and kept at approximately 9°C during transport to the laboratory. The eyes were cleaned of extraocular material, dipped in 0.9% NaCl solution, opened circumferentially approximately 8 mm behind the limbus and the anterior tissues and the vitreous were separated gently from the neural retina. Thereafter the neural retina was gently removed and choroid-RPE was separated from sclera. In total, 94.4 g of choroid-RPE was obtained from 74 eyes. The tissue was dipped in 60 ml of 0.1 M potassium phosphate buffer, pH 8 (PB8) and then stored frozen at −18°C.

Melanin granules were isolated from RPE-choroid using protease digestion (16) followed by sucrose gradient centrifugation (17,18). Amount of 4.3 ml of subtilisin Carlsberg protease type VIII (Sigma-Aldrich, St. Louis, MO, USA) solution (20 mg/ml in PB8) was added into the collected RPE-choroid in PB8. The suspension was divided into two batches and incubated at 56°C for 1 h with frequent stirring. The suspension was heated at 99°C for 15 min, centrifuged (37,000 g, 10 min) and the supernatant was discarded. The pellet was washed with PB8 and centrifuged as above.

The sediment containing melanin granules was suspended in 0.3 M sucrose solution (total volume 50 ml) and layered onto a discontinuous sucrose gradient (1, 1.2, 1.4, 1.5, 1.55, 1.6, 1.8 and 2 M). The overlayered gradient was centrifuged on a swing out head at 103,000 g for 1 h. After the centrifugation, the melanin granules were at the bottom of the tube. The melanin granules were washed with PB8 and centrifuged (37,000 g, 10 min) and the washing procedure was repeated three times. The wet melanin granules were kept at −70°C for 1 h and lyophilized for 18 h (ModulyoD-230, Thermo Savant, Holbrook, NY, USA). After lyophilization the total mass of melanin granules was 1.4 g. Isolated melanin granules were stored at −18°C with a desiccant.

Characterization of Isolated and Synthetic Melanin

SEM Imaging. Samples of isolated and synthetic melanin were coated with gold (Polarin sputter coater 11-E5100, Polaron Equipment Ltd, Watford, UK) and examined using scanning electron microscopy (SEM; XL 30 ESEM TMP, FEI Company, Brno, Czech Republic).

The Specific Surface Area. The specific surface area (SSA) was determined by single-point Brunauer, Emmet, Teller-method (BET; Micromeritics Flowsorb II 2300, Norgross, GA). Before measurement, the samples of isolated and synthetic melanin were dried in vacuum at 37°C for 20 h. The gas in the determination was a nitrogen/helium (70/30%) mixture. A mean of five measurements was used for the calculation of SSA.

Particle Sizing. Suspensions (2 mg/ml) of isolated melanin granules and synthetic melanin (Sigma-Aldrich; prepared by oxidation of tyrosine with hydrogen peroxide) were prepared in purified water, followed by sonication for 15 min. The particle size of melanin granules in the suspensions was measured with a laser diffractometer (Malvern Instruments Ltd, Malvern, UK) immediately after sonication, after 4 h of incubation at 37°C and after 48-h storage at room temperature (the 48 h samples were shaken by hand before measurement).

Binding Experiments

For the experiments with beta blockers and carboxyfluorescein, suspensions (2 mg/ml) of isolated and synthetic melanin were prepared in physiological saline (BSS; Alcon, Fort Worth, TX, USA) containing 50 mM HEPES (Sigma-Aldrich; pH adjusted to 7.4). The suspension was warmed up to 37°C and sonicated for 15 min before incubation with test compounds. For the experiments with oligonucleotides, PBS (Gibco BRL, Grand Island NY, USA) was used as solvent. While being stirred the melanin suspension (0.75 ml) was transferred into a test tube (2.2 ml) and mixed with a test compound solution (0.75 ml; same solvent as above). The polystyrene (for oligonucleotides) or polypropylene (for other compounds) tubes were placed in a shaker (250 rpm; Swip KS-10 Edmund Bühler, Germany) which was kept at 37°C.

The kinetics of the binding of betaxolol [hydrochloride; molecular weight (MW) of 343.9; donated by Alcon, Fort Worth, TX, USA] to isolated and synthetic melanin was determined at two different concentrations (0.1 and 1 μM) using various incubation times (1, 3, 6 and 24 h). The maximum binding capacity and the dissociation constant were determined for betaxolol and metoprolol (tartrate, Sigma-Aldrich) in a concentration range of 0.05 to 100 μM (n=12) using a 4 h incubation time. Because the molecular formula of metoprolol tartrate is $(\text{metoprolol})_2\times\text{tartrate}$ with a MW of 684.8, the molar concentration of metoprolol was calculated using a MW of 342.4 in order to compare the kinetic parameters of betaxolol and metoprolol as free bases. The binding of FITC-labelled 21-mer and 10-mer phosphodiester oligonucleotides (5′-fluorescein-GCC TCG GCT TGT CAC ATC TGC-3′ and 5′-fluorescein-TCA CAT CTG C-3′; Oligomer, Finland) was studied at low concentrations (3 to 28 nM) using 2 h incubations. The binding of 6-carboxyfluorescein (6-CF; Sigma-Aldrich; 20 nM) to melanin was determined with various incubation times (0.5, 1, 5 and 24 h). Controls were prepared by incubating each test compound in pure incubation medium at all the concentrations used in the binding studies. Also controls were prepared by incubating melanin in incubation medium without any test compounds. The number of replicate samples and controls was three to four.

After incubation the suspensions from the beta-blocker and 6-CF experiments were centrifuged (13,000 rpm, 15 min; Centra-M2, International Equipment Company, USA) at 37°C.

The suspensions from the oligonucleotide experiments were centrifuged 5,000 rpm for 15 min (FP-510 centrifuge, Labsystems Oy, Finland). A sample (400–700 μl) of the supernatant was taken for analysis.

Analytic Conditions

Betaxolol and Metoprolol

Betaxolol and metoprolol were determined using HPLC with fluorescence detection (19). The HPLC system consisted of a Beckman system gold programmable solvent module 168 with a model 507e autosampler (Beckman Instruments, Fullerton, CA) with a 100 μl sample loop. A Hewlett–Packard 1046A fluorescence detector (Waldbronn, Germany) was used for detection with excitation at 230 nm and emission at 302 nm. Data processing was performed using 32 Karat software (version 3.0; Beckman Instruments). The separation was carried out on a Kromasil C8 (5 μm, 150×4.6 mm) column (Higgins Analytical, Mountain View, CA, USA) at 30°C. For betaxolol, the mobile phase was acetonitrile/water (35:65, v/v) containing 0.03% (v/v) of trifluoroacetic acid (TFA) and the flow rate was 1 ml/min. The retention time of betaxolol was 6.5 min. For metoprolol, the mobile phase was acetonitrile/water (25:75, v/v) containing 0.03% (v/v) of TFA and the flow rate was 1 ml/min. The retention time of metoprolol was 6.6 min. For each compound, calibration standards were prepared in the concentration range of 0.01 to 100 μM (n=9) in pure incubation medium. The samples with the analyte concentration below 1 μM were determined using the high gain setting of the fluorescence detector to obtain maximum sensitivity (19). Calibration curves were generated by linear regression of peak area to analyte concentration.

Quality control samples at the concentration of 0.1, 1 and 10 μM (n=3 in each batch) were prepared in the supernatant obtained by incubating melanin with incubation medium without any test compounds. The analyte concentration in each control sample was within ±12% of the nominal concentration.

6-CF and Oligonucleotides

6-CF was determined with a 96-well fluorescence plate reader (FL-500, Bio-Tek Instruments, Burlington, VT) with 485 nm excitation and 530 nm emission filters. FITC-labelled oligonucleotides were determined with a 96-well fluorescence plate reader (Victor^2 1420-012, Perkin Elmer–Wallac, Turku, Finland) with excitation at 485 nm and emission at 535 nm. For each compound, calibration standards were prepared in the supernatant obtained by incubating melanin with incubation medium without any test compounds.

Calculation of Binding Parameters

The calculation of binding parameters was based on the Langmuir binding isotherm:

$$B = \frac{B_{\max}[L]}{K_d + [L]}$$

where B is the observed binding of the ligand to melanin (nmol/mg) when the measured unbound (free) concentration of the ligand is equal to $[L]$ (μM), $B_{\max}$ is the maximum binding of the ligand to melanin (nmol/mg), and K_d is the equilibrium dissociation constant for the binding (μM). The results of the binding studies were first inspected visually by preparing Scatchard plots to determine the number of classes of binding sites. For betaxolol and metoprolol, two classes of binding sites were evident. Then, nonlinear fitting based on the Langmuir binding isotherm with two binding sites was performed using Prism software (version 4; GraphPad Software, San Diego, CA, USA) with 1/Y weighting to determine separate binding parameters for the high-affinity site ($B_{\max1}$ and K_{d1}) and the low-affinity site ($B_{\max2}$ and K_{d2}). Individual data points were used in curve fitting.

Prediction of Drug Binding to Melanin in Human Choroid-RPE

The binding of betaxolol and metoprolol to melanin in human choroid-RPE was predicted using the kinetic parameters obtained with bovine ocular melanin. The amount of melanin in human choroid-RPE was assumed to be 7 mg (20) and, therefore, $B_{\max}$ values were calculated per 7 mg of melanin. The amount of melanin bound drug was calculated using the Langmuir binding isotherm with two binding sites. It was assumed that the unbound concentration of the drug is the same in both extracellular and intracellular spaces (complete steady-state conditions). The amount of unbound drug was obtained by multiplying the unbound concentration of drug with the total volume of human RPE-choroid (0.2 ml based on a wet weight of ca. 200 mg; 20). The predictions were made using unbound drug concentrations up to 1,000 ng/ml as betaxolol hydrochloride and metoprolol tartrate. This

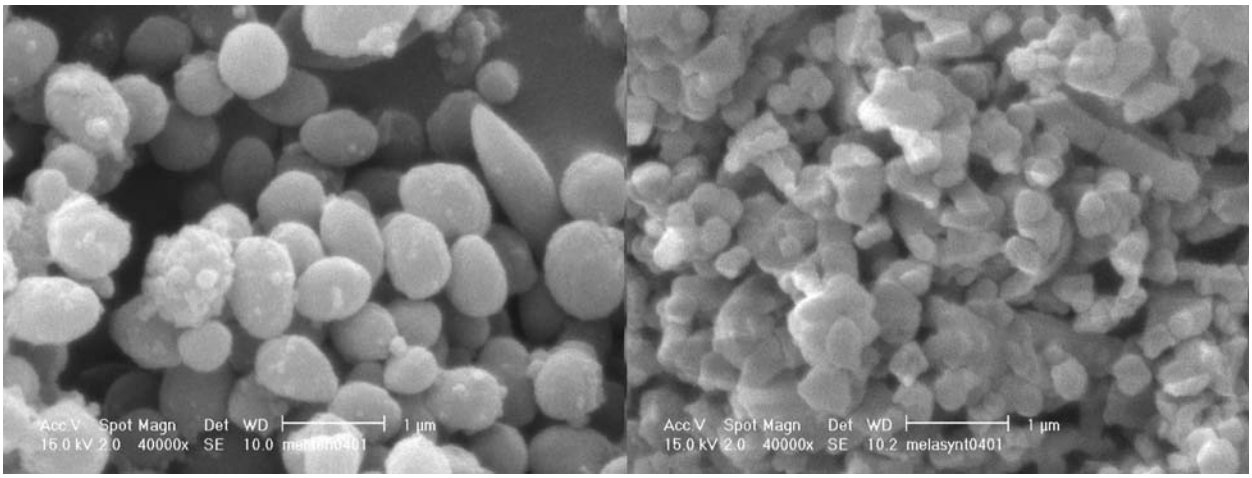

Fig. 1. Scanning electron micrographs of isolated bovine (left) and synthetic (right) melanin granules. The scale bar is 1 µm.

concentration range covers the observed total concentrations of betaxolol in human choroid after long-term topical ocular administration (21) and the observed plasma concentrations of metoprolol after oral administration (typically below 500 ng/ml, unbound fraction is ca. 90%; 22).

RESULTS

Characterization of Melanin Granules

In electron microscopy the bovine melanin granules were round or oval with a diameter of ca.1 µm, whereas the synthetic melanin granules were smaller and irregular in shape (Fig. 1). BET revealed that the specific surface area of bovine and synthetic melanin granules was 59.4 and 117.5 cm^2/mg, respectively. The particle size of melanin granules in aqueous suspensions was determined using laser diffractometry. Immediately after sonication, the diameter of bovine melanin granules varied from 0.2 to 10 µm with a mode at 1.9 µm, and the diameter of synthetic granules ranged from 0.3 to 50 µm with a mode at 1.1 µm, respectively (Fig. 2). After 4 h of incubation there was practically no change in the size of bovine melanin granules, whereas a major proportion of synthetic melanin granules formed larger aggregates with diameters from 10 to 300 µm and a mode at 46 µm (Fig. 2). The bovine melanin granules were also partly aggregated after 48 h of storage at room temperature (data not shown).

Binding of Drugs to Melanin Granules

Betaxolol and Metoprolol

The amount of bound betaxolol (initial concentrations 0.1 and 1 µM) to bovine and synthetic melanin did not change between 1 and 24 h (data not shown). Thus, the equilibrium of binding was achieved within 1 h.

Bovine melanin bound more betaxolol than metoprolol at all ligand concentrations (Fig. 3). Similar results were obtained also with synthetic melanin, but synthetic melanin bound significantly more drug per mass unit of melanin than bovine melanin (Fig. 3). When the amount of bound drug was calculated per surface area of melanin (from BET measurements), the difference between synthetic and bovine melanin was much lower (Fig. 4).

In Scatchard plots, two different types of binding sites are evident for both betaxolol and metoprolol (Fig. 5). The binding parameters obtained by nonlinear fitting are presented in Table I. For betaxolol, the maximum binding

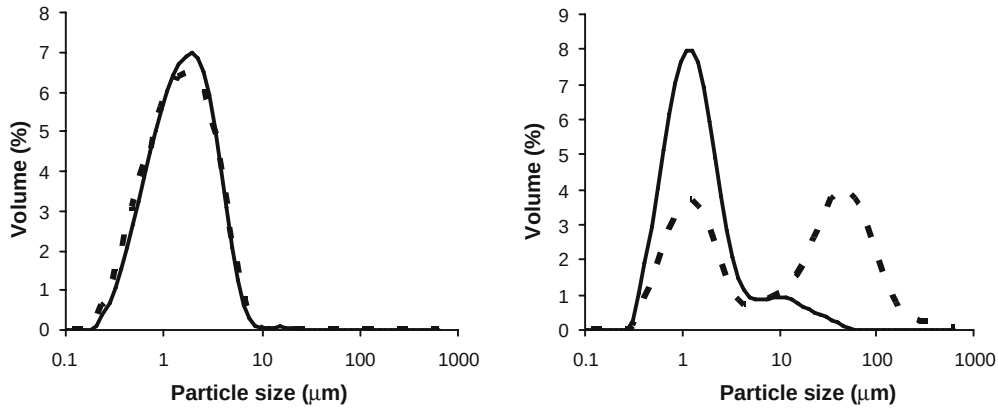

Fig. 2. Particle size distribution of isolated bovine (left) and synthetic (right) melanin granules in purified water immediately after sonication (solid line) and after 4 hr incubation (dotted line).

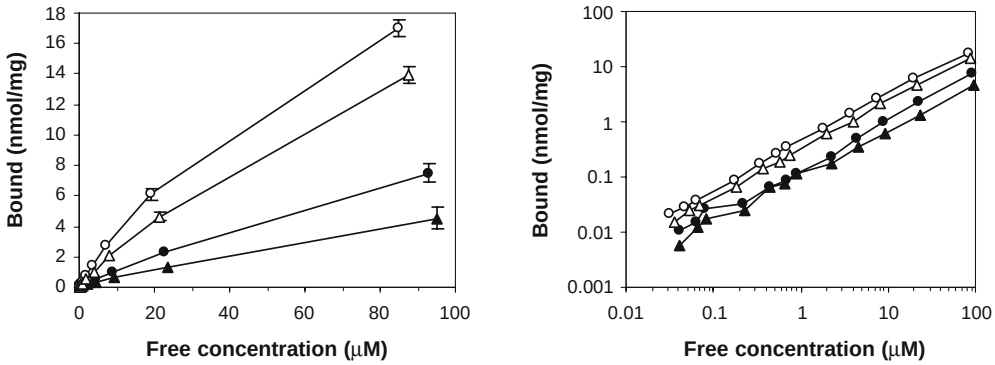

Fig. 3. Binding of betaxolol (circles) and metoprolol (triangles) to isolated bovine (filled symbols) and synthetic (open symbols) melanin granules calculated per mass of melanin using linear scale (left) and log-log scale (right). Error bars indicate standard deviation of 3–4 measurements and are generally encompassed by the symbol. Error bars are not presented on log-log scale.

capacity (B_{max1}) and dissociation constant (K_{d1}) for the high affinity site are much lower in bovine melanin than in synthetic melanin (B_{max1} 0.013 nmol/mg vs 0.152 nmol/mg, K_{d1} 0.026 vs 0.642 μM, respectively). On the other hand, the dissociation constant for the low affinity site (K_{d2}) is much higher in bovine melanin than in synthetic melanin (249 vs 97.6 μM, respectively). The capacity of the low affinity site in bovine melanin is about 2,100 times higher than that of the high affinity site, whereas the corresponding ratio in synthetic melanin is ca. 240. For metoprolol, the binding parameters for the high affinity binding site are fairly similar in both melanin types, whereas the dissociation constant for the low affinity site (K_{d2}) seems to be much larger in isolated melanin.

Weighting by 1/Y was used to improve the fit at low ligand concentrations. The relative residual in the amount of bound drug (residual/mean observed value) was below 16% at low ligand concentrations, and typically between 1 and 5% at high concentrations (data not shown). The exception was the metoprolol data with bovine melanin in which the highest deviation was 28%.

Carboxyfluorescein and Oligonucleotides

Isolated and synthetic melanin did not bind 6-carboxyfluorescein (20 nM) or FITC-labelled phosphodiesther oligonucleotides (3–28 nM; data not shown). Because no change in the concentration of 6-carboxyfluorescein and oligonucleotides was observed after incubation with melanin granules, binding parameters could not be calculated.

Estimation of Drug Binding to Melanin in Human Choroid-RPE

The binding of betaxolol and metoprolol to melanin in human choroid-RPE was predicted assuming that the binding properties of human melanin are similar to bovine melanin. The amount of melanin bound drug increased with the unbound drug concentration (Fig. 6). At the unbound concentration of 1,000 ng/ml (ca. 2.9 μM for both drugs), the amount of melanin bound betaxolol hydrochloride and metoprolol tartrate were 800 and 560 ng, respectively. When the unbound concentration of betaxolol hydrochloride exceeded 37 ng/ml the low-affinity binding site bound more drug than the high-affinity site (data not shown). In the case of the metoprolol tartrate, the corresponding concentration was 320 ng/ml. Within the studied range of unbound concentration, 75–95% of the drug in choroid-RPE was bound to melanin (Fig. 7). This means that the human choroid-RPE was predicted to contain 3–19 times more of melanin bound drug than unbound drug.

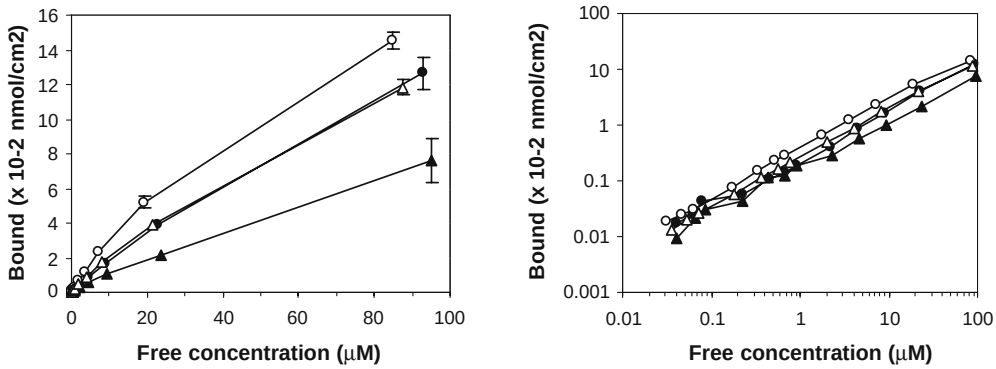

Fig. 4. Binding of betaxolol (circles) and metoprolol (triangles) to isolated bovine (filled symbols) and synthetic (open symbols) melanin granules calculated per surface area of melanin using linear scale (left) and log-log scale (right). Error bars indicate standard deviation of 3–4 measurements and are generally encompassed by the symbol. Error bars are not presented on log-log scale.

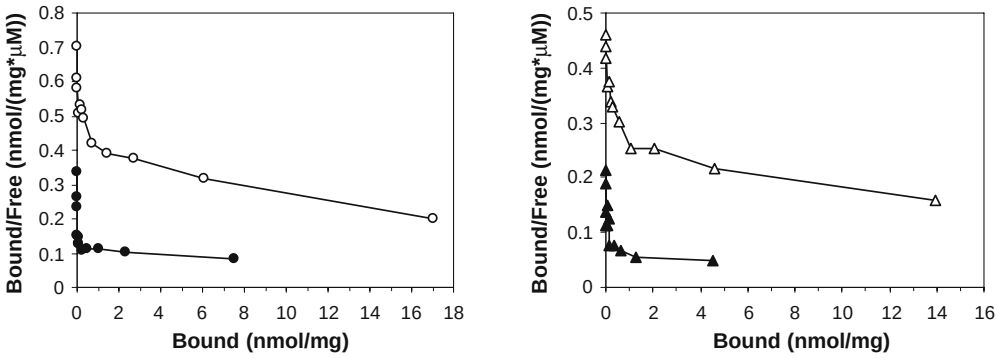

Fig. 5. Scatchard plots for binding of betaxolol (left) and metoprolol (right) to isolated bovine (filled symbols) and synthetic (open symbols) melanin granules.

DISCUSSION

In the present study melanin granules were isolated from bovine choroid-RPE and their characteristics were compared with commercially available synthetic melanin granules. Pure melanin granules were obtained from bovine eyes using protease digestion (16) followed by sucrose gradient centrifugation (17). The shape and size of bovine melanin granules were fairly similar to those isolated from bovine and human RPE by Boulton *et al.* (23). The major melanin component of both human and bovine ocular melanin is eumelanin (3,24).

In this study differences were found between bovine and synthetic melanin granules. Bovine melanin granules were round or oval in shape, whereas synthetic granules were slightly smaller and irregular in shape. The specific surface area of bovine granules was half of that of synthetic granules. Bovine melanin granules did not form aggregates in an aqueous suspension during 4 h incubation, whereas synthetic granules showed marked aggregation, which is a limitation and source of errors. In the aqueous suspension, the diameter of bovine melanin granules varied from 0.2 to 10 μm with an apparent Gaussian distribution and a mode at 1.9 μm. Earlier, Koeberle *et al.* (15) described that in an aqueous suspension a significant fraction of melanin granules isolated from bovine eye had a diameter above 100 μm. A possible explanation for the observed difference in the diameter of granules is that Koeberle *et al.* (15) did not use sucrose gradient centrifugation in the purification of melanin granules unlike in the present study.

There were differences between bovine and synthetic melanin granules also in their ability to bind betaxolol and metoprolol. Bovine melanin bound less drug (per unit of mass or surface area) than synthetic melanin. It is important to normalize binding to the surface area because the binding is related to the number of binding sites and their availability (i.e. surface area). Structural dissimilarities between natural and synthetic melanins have already been reported: Ito *et al.* (1) reported that enzymatically prepared dopa melanin contains a lower percentage of 5,6-dihydroxyindole-2-carboxylic acid (DHIC)-derived monomers than natural eumelanin. The natural eumelanin from *Sepia officinalis* has a structural order with subunits that have a lateral dimension of ~15 nm while the synthetic eumelanin appears to be amorphous solids (14).

At least two binding sites were evident for both betaxolol and metoprolol, which is a typical feature for beta-blockers and many other drugs (13,25). For betaxolol the high and low affinity binding sites were markedly different in bovine and synthetic melanin. The maximum binding capacity (B_{max1}) and dissociation constant (K_{d1}) of the high-affinity site were much lower in bovine melanin than in synthetic melanin, whereas the dissociation constant of the low-affinity site (K_{d2}) was much higher in bovine melanin. Also for metoprolol the dissociation constant of the low-affinity site (K_{d2}) in bovine melanin seemed to be much higher than in synthetic melanin. Earlier, Koeberle *et al.* (15) compared bovine, sepia and synthetic melanin in binding memantine, a basic drug candidate with an aliphatic structure. A low-affinity binding site (K_d above 300 μM) was observed in each melanin type, but there were marked differences in B_{max} and K_d values. The K_d of memantine in isolated bovine melanin was higher than in synthetic melanin (804 versus 442 μM) in a phosphate buffer solution. In the present study similar results were obtained for the low-affinity binding site of betaxolol and metoprolol.

In the present study neither isolated nor synthetic melanin bound FITC-labelled phosphodiesther oligonucleo-

Table I. Nonlinear Fitting of Data to Langmuir Binding Isotherm with Two Binding Sites[a]

	Betaxolol		Metoprolol	
	Bovine Melanin	Synthetic Melanin	Bovine Melanin	Synthetic Melanin
B_{max1} (nmol/mg)	0.013 (0.009)	0.152 (0.064)	0.096 (0.057)	0.166 (0.062)
K_{d1} (μM)	0.026 (0.069)	0.642 (0.375)	0.832 (0.744)	0.915 (0.446)
B_{max2} (nmol/mg)	27.5 (3.6)	36.3 (1.3)	26.9 (15.6)	40.7 (2.6)
K_{d2} (μM)	249 (41)	97.6 (5.9)	495 (340)	172 (16)
R^2	0.994	0.998	0.977	0.998

[a] Data are expressed as the best fit value with the standard error in parentheses. R^2 is the coefficient of determination.

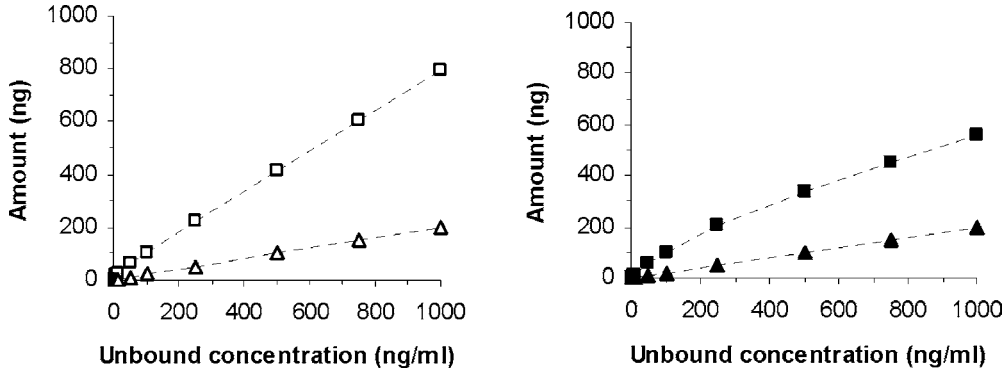

Fig. 6. Predicted amount of melanin bound (squares) and unbound (triangles) betaxolol hydrochloride (left) and metoprolol tartrate (right) in human RPE-choroid based on the binding studies with isolated bovine melanin.

tides or 6-carboxyfluorescein which contain negatively charged functional groups. This is not surprising since melanin contains negatively charged groups that repel compounds with the same charge. We also made preliminary experiments with FITC-labelled phosphorothioate oligonucleotides, but the results were not reliable due to decrease of fluorescence in the control samples possibly because of adherence to the tubes or micelle formation. When binding experiments with phosphorotioate oligonucleotides were done in physiological saline containing 4% of ethanol, the results were reliable and no binding to melanin was seen. However, ethanol may hamper the binding process. Oligonucleotide drugs are known to accumulate into retina and RPE after intravitreal injection (26–28). Our results suggest that the accumulation into RPE is not due to melanin binding.

In drug therapy, the binding of drugs to melanin in choroid-RPE may affect drug transfer through these tissues after systemic, transscleral (e.g. a periocular injection or an implant on the scleral surface) or intravitreal administration. Recently, bovine and porcine choroid-Bruch's layer was found to hinder the in vitro transport of lipophilic solutes more than hydrophilic solutes and the reduction in transport directly correlated with solute binding to the tissue (29). It was suggested that the lipophilic compounds bound to melanin, lipidic plasma membranes and proteins. In addition, after a subconjunctival injection, the exposure (the area under the concentration curve) of celecoxib in choroid-RPE was higher in pigmented rats than in albino rats, whereas the exposure in retina and vitreous was higher in the albino rats (30). We have found earlier that the permeation lag time of lipophilic beta-blockers (e.g. betaxolol and metoprolol) through bovine RPE-choroid was ca. 100 min, whereas the lag time of 6-carboxyfluorescein was ca. 30 min (11). In the present study betaxolol and metoprolol bound to isolated bovine melanin, whereas 6-carboxyfluorescein did not. This suggests that melanin binding may contribute to the observed permeation lag time of the lipophilic beta-blockers. The steady-state flux through the tissue begins only after the equilibrium of drug binding in the melanosomes has been achieved.

Melanin binding may also lead to the accumulation of drug in the choroid-RPE and this may prolong the action of the drug. We predicted the binding of betaxolol and metoprolol to melanin in the human choroid-RPE using the binding parameters obtained with bovine ocular melanin. It seems that melanin in the human choroid-RPE may bind even hundreds of nanograms of betaxolol and metoprolol at therapeutic drug concentrations. Based on our predictions the amount of melanin bound drug is 3 to19 times higher than the amount of unbound drug. Recently, Hollo *et al.* (21) found a high concentration of betaxolol (1,290±1,170 ng/g) in the human choroid after 1 month of topical ocular administration. A very high concentration of betaxolol (73,200±89, 600 ng/g) was also found in the iris, another melanin-containing tissue. It is important to further clarify the role of melanin binding in the accumulation and ocular pharmacokinetics of betaxolol in the RPE.

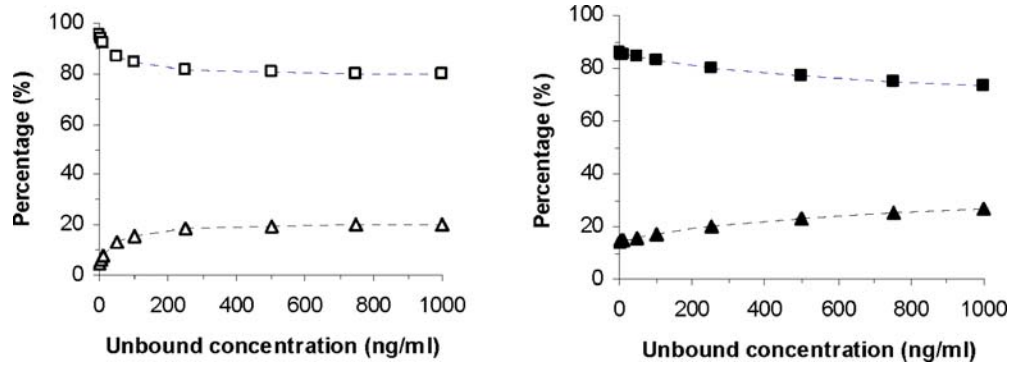

Fig. 7. Predicted percentage of melanin bound (squares) and unbound (triangles) betaxolol hydrochloride (left) and metoprolol tartrate (right) in human RPE-choroid based on the binding studies with isolated bovine melanin.

The prediction of drug binding in the human choroid-RPE is based on the data on bovine melanin granules. Although the morphology of human and bovine melanin granules is fairly similar in electron microscopy (23) and eumelanin is the major component in both human and bovine melanin (3,24), there may be differences in their binding properties. Because of the poor availability of human ocular melanin, there are only limited data on its binding properties. Dayhaw Parker *et al*. (31) studied the binding of chlorpromazine and chloroquine to melanin granules from human RPE, but the experimental conditions are not given in their published abstract. The binding of timolol to human iris-ciliary body melanin was studied by Menon *et al*. (32). However, they did not use intact melanin granules, but granules were hydrolyzed in hydrochloric acid solution. Therefore, more information of the binding properties on human melanin is needed.

In conclusion, the binding of lipophilic drugs to biological melanin differs from that of synthetic melanin. Biological melanin should be used for accurate binding characterization. Kinetic calculations based on our data suggest that melanin in human choroid-RPE binds a remarkable amount of lipophilic beta-blockers. This may prolong the lag time in drug permeation and cause accumulation of the drug in these pigmented tissues. The tendency of oligonucleotides to accumulate to the RPE does not seem to be caused by melanin binding.

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