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Melanin binding study of clinical drugs with cassette dosing and rapid equilibrium dialysis inserts

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Abstract

Melanin pigment is a negatively charged polymer found in pigmented human tissues. In the eye, iris, ciliary body, choroid and retinal pigment epithelium (RPE) are heavily pigmented. Several drug molecules are known to bind to melanin, but larger sets of drugs have not been compared often in similar test conditions. In this study, we introduce a powerful tool for screening of melanin binding. The binding of a set of 34 compounds to isolated porcine RPE melanin was determined by cassette (n-in-one) dosing in rapid equilibrium dialysis inserts and the binding was quantitated with LC-MS/MS analytics. The compounds represented large variety in melanin binding (from 8.6 %, ganciclovir) to over 95 % bound (ampicillin and ciprofloxacin). The data provides information on melanin binding of small molecular weight compounds that are used for ocular (e.g. brinzolamide, ganciclovir) and systemic (e.g. tizanidine, indomethacin) therapy. Interestingly, competition among compounds was seen for melanin binding and the binding did not show any correlation with plasma protein binding. These results increase the understanding of melanin binding of ocular drugs and can be further exploited to predict pharmacokinetics in the eye. Pigment binding provides an interesting option for improved drug distribution to retina and choroid that are difficult target tissues in drug delivery.
1 Introduction

Melanin is a polyanionic polymer synthesized inside specialized intracellular organelles, melanosomes (Ito, 1986; Różanowska, 2011). Melanosomes are found in pigmented tissues including skin, hair, inner ear and the iris, choroid and retinal pigment epithelium (RPE) in the eye. In the RPE, melanin absorbs scattered light and protects the neural retina against solar radiation and enables sharp vision (Colombo et al., 2011; Różanowska, 2011). Melanosomes also maintain cellular homeostasis of many metals (Fe, Ca, Zn, Mg and Cu) (Hong and Simon, 2007; Kaczara et al., 2012) and detoxify free radicals to protect the neural retina (Burke et al., 2011). In addition, melanin is known to bind several drug molecules (Larsson, 1993; Leblanc et al., 1998). For instance, atropine accumulates into pigmented tissues (iris, uvea) after topical administration leading to prolonged drug action (Salazar and Patil, 1976). Furthermore, evidence on the importance of pigment binding in pharmacokinetics and pharmacodynamics in the posterior eye has been presented (Robbie et al., 2013). Recent experiments and follow-up simulations showed that pigment binding plays an important role in the drug distribution to the RPE (Rimpela et al., 2016). Even though binding of drugs to melanin has been demonstrated since 1964 (POTTS, 1964b), the mechanisms of binding remains still poorly understood.

During the last decades, several reports describe the melanin binding of compounds with various assay conditions (Leblanc et al., 1998). Chemicals binding to melanin include clinical drugs from several different therapeutic groups (e.g. antibiotics, beta blockers, antipsychotics) but also dyes, herbicides, alkaloids and metals (Dayhaw-Barker, 2002; Larsson, 1993; Leblanc et al., 1998). However, reported binding percentages cannot be compared between different studies, because this parameter is sensitive to alterations in the assay protocols. For instance, fraction of drug bound to melanin depends on the drug concentration in the assay: the bound fraction is high at low drug concentrations and decreases at higher drug concentrations (Rimpela et al., 2016). Fundamental binding parameters, such as binding affinity (i.e. dissociation constant \( K_d \)) and maximal binding capacity (\( B_{\text{max}} \)) are comparable among different studies, but to calculate these parameters, melanin binding at several drug concentrations needs to be determined. In addition, different isotherms have been used to calculate binding at equilibrium, which makes the comparison between reported \( K_d \) and \( B_{\text{max}} \) values difficult. The binding equilibrium is traditionally calculated from the Langmuir isotherms using one (Pescina et al., 2012) or two (Pitkanen et al., 2007) binding sites, but recently Sips model was shown to describe melanin binding more realistically, since it is not based on specific binding sites but rather on charged surface where compounds can move (Manzanares et al., 2016; Rimpela et al., 2016).

Pigment binding experiments should be done in controlled, specified and constant conditions to reliably distinguish differences in the melanin binding of compounds. Traditionally, melanin binding assays are conducted by letting the isolated or synthetic melanin interact with the compound of interest in regular microcentrifuge tubes or multi-well plates. This approach requires removal of melanin by centrifugation, and the free drug concentration is measured from the resulting supernatant. Furthermore, earlier studies often describe the binding of very few compounds, allowing only limited comparisons of different compounds. In this paper, we describe a method based on cassette (n-in-one) dosing and rapid equilibrium dialysis inserts (RED) to study melanin binding in vitro in a simple and robust way. We utilize porcine RPE melanin and a cassette set of clinical drugs displaying large chemical space. RED inserts provide a platform where melanin removal is not needed to study the unbound fraction (Fig. 1). These inserts are commonly used in plasma protein binding assays, but they have not been used previously to study pigment binding of drugs.

2 Materials and Methods

2.1 Melanin isolation
The RPE cells were isolated as described previously (Pelkonen et al., 2016). Briefly, porcine eyes were obtained from a local slaughterhouse and the eyes were kept in phosphate buffered saline (PBS) during the transportation. The extraocular material was removed followed by the removal of the anterior part of the eye. The vitreous and neural retina were detached and PBS buffer was added into the eyecup. After 5 min incubation at room temperature, RPE cells were gently removed using a small paintbrush and collected. Centrifugation at 6238 g for 5 min was used to pellet the cells. Cell pellets were stored at –20 °C until further processing.

The RPE cells were thawed on ice and lysed using hypotonic buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM MgCl₂) and nitrogen cavitation (Parr 4639, Parr Instrument Co., Moline, IL, USA) at 450 psi with 15 min equilibration time. The cell lysate was centrifugated at 3000 g for 5 min at +4 °C resulting in crude melanosomal pellet (Pelkonen et al., 2016). For the melanin isolation, the supernatant was removed and the crude melanosomal fraction was introduced to hypo-osmotic shock with 10 mM HEPES and treated with nitrogen cavitation at 1500 psi for 15 min to disrupt the organelles. The disruption of the crude melanosomal fraction was completed using sonication (23 % amplitude, 2 x 15 seconds, SONICS Vibracell VCX750 Ultrasonic Cell Disrupter) and melanin was pelleted at 10 000 g 5 min at +4 °C. The supernatant was removed and the melanin pellet was washed with milli-Q-H₂O and sonicated (2 x 15 s), pelleted (10 000 g 5 min at +4 °C) and pre-cooled for 30 min at -80 °C before lyophilization for 48 h (ModulyoD-230, Thermo Savant, Holbrook, NY, USA). Dry melanin was weighed and stored at –20 °C until further use.

### 2.2 Cassette mix preparation

In a single cassette mix assay, 34-37 drug molecules were studied simultaneously. The stock solution of each compound was prepared into either in PBS or DMSO (Sigma Aldrich) at concentration of 1 mg/ml (Table 1). The cassette drug mix was prepared by combining the compounds into one solution and diluting it with PBS (pH 7.4) to a final concentration of 550 ng/ml. The DMSO concentration in the assay was below 0.5 % (below 0.2 % at equilibrium).

**Table 1. Compound information melanin binding assays**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>Cat n:o</th>
<th>Manufacturer</th>
<th>Compound</th>
<th>Solvent</th>
<th>Cat n:o</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyclovir</td>
<td>DMSO</td>
<td>A-4669-500MG</td>
<td>Sigma-Aldrich</td>
<td>Indomethacin</td>
<td>DMSO</td>
<td>I-7378</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Acetazolamide</td>
<td>PBS</td>
<td>A6011-10g</td>
<td>Sigma-Aldrich</td>
<td>Ketorolac</td>
<td>PBS</td>
<td>K1136-1G</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>PBS</td>
<td>A9518-5g</td>
<td>Sigma-Aldrich</td>
<td>Levozastaine</td>
<td>DMSO</td>
<td>L3042-5mg</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Atenolol</td>
<td>PBS</td>
<td>A7655</td>
<td>Sigma-Aldrich</td>
<td>Lincomycin</td>
<td>PBS</td>
<td>31727-250mg</td>
<td>Fluka</td>
</tr>
<tr>
<td>Atropine</td>
<td>PBS</td>
<td>11330</td>
<td>Fluka</td>
<td>Lornxicam</td>
<td>DMSO</td>
<td>SML0338-10mg</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>DMSO</td>
<td>A6848-50MG</td>
<td>Fluka</td>
<td>Methasolamide</td>
<td>DMSO</td>
<td>37011-100mg</td>
<td>Fluka</td>
</tr>
<tr>
<td>Betaxolol</td>
<td>PBS</td>
<td>0222648</td>
<td>Alcon Belgium</td>
<td>Methotrexate</td>
<td>DMSO</td>
<td>PHR1396-1g</td>
<td>Fluka</td>
</tr>
<tr>
<td>Brinzolamide</td>
<td>DMSO</td>
<td>SML0216-10mg</td>
<td>Sigma-Aldrich</td>
<td>Nadolol</td>
<td>PBS</td>
<td>N-1892</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Bromfenac</td>
<td>PBS</td>
<td>SML0289-10mg</td>
<td>Sigma-Aldrich</td>
<td>Penicillin G</td>
<td>PBS</td>
<td>P-7794</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Carteolol</td>
<td>PBS</td>
<td>Charge3849</td>
<td>Dr.Madaus&amp;Co</td>
<td>Pilocarpine</td>
<td>PBS</td>
<td>5034680</td>
<td>Santen Oy</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>DMSO</td>
<td>C4895</td>
<td>Sigma-Aldrich</td>
<td>Pindolol</td>
<td>PBS</td>
<td>P-0778</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>PBS</td>
<td>C6628</td>
<td>Sigma-Aldrich</td>
<td>Prednisolone</td>
<td>DMSO</td>
<td>102717</td>
<td>MP Biomedicals</td>
</tr>
<tr>
<td>Chlormethiazol</td>
<td>PBS</td>
<td>C4830</td>
<td>Sigma-Aldrich</td>
<td>Prednisolone</td>
<td>DMSO</td>
<td>102717</td>
<td>MP Biomedicals</td>
</tr>
</tbody>
</table>
Melanin binding assays

Melanin binding studies were performed in RED inserts with 8000 Da molecular weight cut-off (#89809, Thermo Fisher Scientific, Waltham, MA USA) in a re-usable base plate (#89811, Thermo Fisher Scientific). Three replicate inserts were used for each assay with different melanin concentrations (Assay 1: 0.5 mg/ml, Assay 2: 1 mg/ml, Assay 3: 5 mg/ml and Assay 4 and 5: 10 mg/ml) and another three inserts without melanin were used as controls (Assays 1-5, Table 2). The assays were conducted at + 37 °C on a horizontal shaker at 220 rpm (Heidolph incubator 1000 and Titramax 1000, Heidolph Electro GmbH & Co., Keiheim, Germany). All the reagents in the binding assay were pre-warmed at + 37 °C before the experiment was started.

Table 2. Detailed information regarding the melanin binding assays 1-5.

<table>
<thead>
<tr>
<th>Melanin concentration (donor chamber)</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
<th>Assay 4</th>
<th>Assay 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mg/ml melanin</td>
<td>Eq. 2</td>
<td>Eq. 2</td>
<td>Eq. 2</td>
<td>Eq. 2</td>
<td>Eq. 1</td>
</tr>
<tr>
<td>1 mg/ml melanin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/ml melanin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/ml melanin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drugs in the assay</td>
<td>Cassette drug mix (37 compounds)</td>
<td>Cassette drug mix (37 compounds)</td>
<td>Cassette drug mix (37 compounds)</td>
<td>Cassette drug mix (34 compounds)</td>
<td>Individually incubated compounds (9 compounds)</td>
</tr>
<tr>
<td>Sample collection</td>
<td>1 h, 2 h, 4 h, 6 h and 10 h</td>
<td>1 h, 2 h, 4 h, 6 h and 10 h</td>
<td>1 h, 2 h, 4 h, 6 h and 10 h</td>
<td>8 h and 10 h</td>
<td>10 h</td>
</tr>
<tr>
<td>Equation for binding determination</td>
<td>Eq. 2</td>
<td>Eq. 2</td>
<td>Eq. 2</td>
<td>Eq. 2</td>
<td>Eq. 1</td>
</tr>
</tbody>
</table>

Melanin was suspended into PBS (pH 7.4) at desired concentration (1 mg/ml; 2 mg/ml; 10 mg/ml and 20 mg/ml), sonicated (23 % amplitude, 2 x 15 seconds, SONICS VibraCell VCX750 Ultrasonic Cell Disrupter) and pipetted into the donor chambers of the RED inserts (V=100 µl) (Fig. 1). The cassette drug mix (550 ng/ml of each drug, 100 µl) was added to the donor chamber and PBS (pH 7.4, 350 µl) to the receiver channel immediately afterwards (Fig. 1). The plate was covered with a sealing tape and transferred onto the shaker.
At each time point, 30 µl of sample was collected from the buffer compartment and the sample volume was replaced with 30 µl of warm PBS (pH 7.4). Samples were collected at 1 h, 2 h, 4 h, 6 h and 10 h in the assays 1-3 (0.5 mg/ml; 1mg/ml and 5 mg/ml melanin) and at 8 h and 10 h in the assay 4. In the assay 5 where 9 compounds were incubated individually with melanin, samples were collected only at 10 h (Table 2). In all studies, the starting concentration of the drug mix was also quantified. The samples were stored at –80 °C until further processing.

Figure 1. Graphical representation of the melanin binding assays. Melanin suspension (c= 1-20 mg/ml, V=100 µl, pH 7.4) and drug solution (c=550 ng/ml in PBS, pH 7.4) were inserted into the donor chamber of the RED insert. PBS at pH 7.4 was added to the receiver chamber. Samples were collected from the receiver chamber.

2.4 Sample preparation for LC-MS/MS

Internal standard mixture (atenolol-d7, atropine-d5, cephalexin-d5, dexamethasone-d5, fluconazole-d4, ganciclovir-d5, indomethacin-d4, lincomycin-d3, lornoxicam-d4; Toronto Research Chemicals Inc and methotrexate-d3, Sigma Aldrich) was added into each sample (final concentration 7 ng/ml) (Ramsay et al., 2017) and the samples were transferred to the inserts for LC-MS/MS analysis in 1.5 ml vials (Agilent Technologies). Internal standard solution was prepared in 30 % acetonitrile. Standard and quality control samples were prepared from the drug mixture used in the assays (Table 1).

2.5 LC-MS/MS analysis

The samples were analyzed by positive ion electrospray LC-MS/MS using an Agilent 6495 triple quadrupole mass spectrometer coupled with Agilent 1290 liquid chromatograph (Agilent Technologies Inc., USA). UHPLC separation was done on an Agilent Poroshell® 120 SB-C18 column (2.7 µm, 2.1 x 50 mm) maintained at 50 °C at a flow rate of 0.3 ml/min. Eluent A was 0.1 % formic acid (eluent additive for LC-MS, Fluka) in milliQ-H2O and eluent B was methanol (Ultra Chromasolv for LC-MS, SigmaAldrich). The following gradient was employed: eluent B was kept at 2 % for the first 2 minutes, followed by an increase to 100 % in 10 minutes (total run time 16 minutes). The injection volume was 2 µl. MRM mode was employed for data acquisition, monitoring two product ions for each compound (quantifier and qualifier). The MS conditions were the following: capillary voltage 3.5 kV, nebulizer 25 psi, gas temp 200 °C, gas flow 16 l/min, sheath gas heater 400 °C, sheath gas flow 11 l/min, Fragmentor voltage 380 V, dwell time 20 ms, cell accelerator voltage 5 V. Collision energy was optimized for each ion, and energies varying from 4 to 50 V were used. Divert valve was used to direct the eluent flow to waste except during data acquisition, and the program was divided into segments according to the compounds’ retention times (early-eluting, middle-eluting, and late-eluting). Agilent MassHunter Quantitative Analysis software was used for data analysis.

The calibration curves were calculated as a mean of two injections (beginning and end of each analysis). The attainable lower limit of quantitation (LLOQ) and curve fitting varied between compounds; in general, quadratic fitting with 1/x weighting was usually the best fit. Each analysis was accepted or rejected depending on the calibration curve and triplicate quality control (QC) standards, according to European Medicines Agency guideline criteria (EMA 2009). Curve acceptance criteria included a maximum residual error of ≤ 15 %, a correlation coefficient of ≥ 0.99 and a minimum of six concentration levels with accuracies within the guideline limits. Selectivity criteria (a minimum 5x response ratio of LLOQ to matrix-based blank sample) were employed as well. The internal standards were not used in calculations at this time, since their optimization is still ongoing and they were not essential due to minimal sample preparation. The calibration and QC standards were matrix-based, diluted with PBS buffer as were the actual samples. A partial validation analysis was performed in a similar manner but injecting QC standards and separate LLOQ standards in five parallels, employing the same acceptance criteria. The validation was successful for 26 compounds (see Supplementary information, Table S2). Carryover was minimized by...
avoiding sample randomization and by injecting neat blank samples (acetonitrile) after high-concentration standards; furthermore, significant carryover was not observed for most compounds (max. 0.63 %, calculated as response of the first neat blank after high-concentration standards, % of the preceding high-concentration standard response).

2.6 Melanin binding calculations

Melanin binding values were determined with the equations below. These equations consider also the non-specific binding; thus, the binding percentages represent only the melanin bound fraction.

Equation 1: \[
\text{\% Bound}_{\text{melanin}} = (1 - \frac{C_{\text{melanin}, 10 \text{ h}}}{C_{\text{blank}, 10 \text{ h}}}) \times 100
\]

or

Equation 2: \[
\text{\% Bound}_{\text{melanin}} = \text{\% Bound}_{\text{total}} - \text{\% Bound}_{\text{background}}
\]

Where

\[
\text{\% Bound}_{\text{total}} = (1 - \frac{\text{Free Amount}_{\text{melanin}}}{\text{Total Amount in the system}_{\text{melanin}}}) \times 100
\]

\[
\text{\% Bound}_{\text{background}} = (1 - \frac{\text{Free Amount}_{\text{blank}}}{\text{Total Amount in the system}_{\text{blank}}}) \times 100
\]

Where

Free amount = Concentration * Volume of the system

Amount in the system = Amount inserted – Amount removed

3 Results

3.1 The drug mix equilibrium between the RED chambers

The compounds in the drug mix reached equilibrium in the donor and receiver chambers after 4 h incubation (data regarding selected compounds presented in Fig. S1), as expected based on the RED insert instruction manual (Thermo Fischer Scientific). The drug concentration levels in the donor and receiver chambers were similar in blank inserts at the 10 h time point confirming that equilibrium had been reached between the chambers at the end of the assay (Fig. S2).

3.2 Melanin binding at equilibrium

Melanin binding reached equilibrium at approximately at 6-8 h (data not shown). The equilibrium binding calculations were done based on the binding after 10 h incubation. The quantitation of cephalexin, chloroquine and streptomycin was not successful in the 0.5 mg/ml; 1 mg/ml and 5 mg/ml melanin binding assays, and thus they were excluded from the following experiments (Table 1 and 2). For one drug (Iornoxicam), melanin binding could not be determined due to analytical problems in the 10 mg/ml melanin binding assay with drug mix (Table 1).

In the cassette dosing assays, melanin binding percentages increased as the melanin amount in the assay increased (Fig. S3). However, the binding followed similar rank order regardless of the melanin amount: the highest binders displayed the highest binding percentages in each assay and lowest binders similarly regardless of the melanin concentration (Fig. S3).

When the melanin amount in the cassette assay was increased to 10 mg/ml, (assay 4:10 mg/ml melanin, Table 2), melanin binding of 31 compounds was determined (Fig. 2). Two compounds (fluconazole and methazolamide) did not bind to melanin and the remaining compounds represented a wide range of melanin binding and could be classified into low binders (below 50 %), intermediate (50-80 %) and high (80-
100 %) binders (Fig. 2). The majority of the compounds were classified as low binders (19/31), whereas fewer compounds displayed intermediate or high binding (6/31 compounds in each group) (Fig. 2).

**Figure 2. Melanin binding values (%) in the assay 4 consisting of cassette drug mix (34 compounds) and high melanin concentration (10 mg/ml).** Black bars represent low binders (below 50 %), light blue bars intermediate binders (50-80 %) and red bars high binders (over 80 %). Fluconazole and methazolamide did not bind to melanin.

To determine, whether compounds might compete for melanin binding, we carried out tests using individual compounds incubated with 10 mg/ml of melanin. In these experiments 9 compounds (methotrexate, methazolamide, bromfenac, indomethacin, nadolol, atropine, tetracycline, tizanidine and ciprofloxacin) were studied individually (Table 1 and 2). The binding percentages differed from those determined in the cassette study with the same melanin amount, but the classification remained same (Fig. 2 and 3, Table S1).

**Figure 3. Influence of assay conditions for melanin bound drug fraction.** Black bars represent the assays conducted with cassette mix (37 drugs) and lower melanin concentrations (0.5-5 mg/ml). Red bars represent the assays with the highest melanin concentration (10 mg/ml): cassette assay with 34 drugs and incubation individually without other compounds.

**4 Discussion**

In this paper, we introduce a novel method to screen melanin binding of compounds according in commercially available RED inserts and describe melanin binding values for a small molecular weight compound set of clinical drugs. Simultaneous analysis of 34 compounds using two MRM reactions is a specific, efficient screening tool with multiple applications in ocular drug research (Ramsay et al., 2017). With RED inserts, samples can be collected without melanin removal. The dialysis membrane between chambers enables the diffusion of compounds below 8000 Da, while larger compounds (such as melanin) do not permeate through the dialysis membrane, resulting in equal free drug concentration in the donor and receiver chambers (Fig.1, Thermo Fisher Scientific). Cellophane dialysis membrane has been adapted to melanin binding assays successfully, however, this study described the binding of one compound (clindamycin) (Barza et al., 1979). Previously, melanin containing chromatographic columns have been used for melanin binding screens (Reilly et al., 2015). Our binding assay method lacks the capacity of chromatographic method, but it maintains melanin in its native state and can be easily set-up to melanosome binding experiments.

We used physiological pH (7.4) in the melanin binding assays. It has been suggested that melanosomes have acidic intraorganellar pH (Bhatnagar et al., 1993), but that study involved melanoma melanosomes. The pH inside ocular melanosomes is not known. The pH should be determined in ocular melanosomes, since compounds and melanin itself may represent different ionization states at various pH values (Mani et al., 2001; Rimpela et al., 2016), thereby leading to pH dependent melanin binding levels (Rimpela et al., 2016; Tsuchiya et al., 1987). Further investigation is needed to apply the most relevant conditions for *in vitro* melanin binding assays.

In our study, 32/34 compounds bound to melanin (Fig. 2 and 3) and a large range in binding was seen: from 8.6 % to over 95 % (Fig. 2). Furthermore, a categorization to low, intermediate and high binders (below 50 %, 50-80 % and above 80 %, respectively) was possible (Fig. 2). Some of these clinical drugs are known to bind to melanin (e.g. atropine, pilocarpine, betaxolol), but for the majority of the compounds (18/34), melanin binding has not been studied earlier. These drugs are acetazolamide, atenolol, aztreonam, brinzolamide, bromfenac, cephalaxin, diclofenac, fluconazole, fluoromethalone, ketorolac, levocabastine, lincomycin, lornoxicam, prednisolone, tizanidine, voriconazole, ampicillin and dexamethasone. Earlier literature suggests that basic and lipophilic compounds can be expected to bind to melanin (Leblanc et al., 1998). Our compound library displayed large variety in physicochemical properties (Supplementary
information, Ramsay et al., 2017), including both acidic (e.g. penicillin, indomethacin, diclofenac, bromfenac) and basic compounds (e.g. propranolol, lincomycin, brinxolamide, pilocarpine) with variable lipophilicity (logD$_{7.4}$ values ranging from -5.1 to 4.2). For instance, penicillin displayed high binding (Fig. 2, Table S1), but is acidic (pKa 2.45) and hydrophilic (logD$_{7.4}$-1.81). Thus, lipophilicity and basic nature are not the only factors affecting melanin binding.

Incubation of individual compounds resulted in higher binding as compared to the values in the cassette mix (Fig. 3) suggesting that there is competition for the binding on the melanin surface. While the binding values were changed, the binding classification (low, intermediate, high) remained the same (Fig. 3). Even though the cassette approach does not yield exact binding percentages, it can be used for compound categorization and screening of melanin binding (Fig. 2 and 3, Table S1). The earlier literature suggests that melanin binding has high capacity, it is non-specific and the binding in general has low affinity (Manzanares et al., 2016). Thus, it was surprising that even at high melanin amounts some competition for binding was seen (Fig. 3). The drug concentrations were relatively low in the assay (0.1 µg/ml; 0.22-0.48 µM) compared to the reported dissociation constants (K_d) for melanin binding that vary typically at micromolar levels (100-350 µM; (Pescina et al., 2012; Pitkanen et al., 2007; Rimpela et al., 2016). However, depending on the binding isotherm used, K_d values lower than 1 µM are also reported (Pescina et al., 2012).

Competition for the binding sites on melanin surface has been recognized earlier in the field. Already in the 1960’s, Potts showed that chlorpromazine adsorption on pigment was 4.2-fold lower when pigment was pre-treated with acridine orange (POTTS, 1964a). Other examples include lower binding of haloperidol to melanin in the presence of desipramine (Knorle et al., 1998) and chlorpromazine (Ibrahim and Aubry, 1995). Thus, if melanin binding experiments are conducted using several compounds simultaneously, the role of competition should be investigated. The largest differences in melanin binding between cassette assay and individual compounds were observed with low or intermediate binders, whereas the binding percentages of the high binders (tizanidine, ciproflaxacin) did not change dramatically (Fig. 3). However, free drug is pharmacologically active, and therefore, changes in free drug concentration would be the most relevant parameter to describe the impact on drug response. When only a small fraction of the compound is in the free form, the relative change in the free drug concentration is large. For instance, the bound fraction of ciproflaxacin was over 98.5 % when incubated alone with melanin. This corresponded to less than 2.5 % of free drug. In the cassette assay, the bound fraction was decreased to 95.6 %, corresponding 4.4 % of unbound fraction. This lead to over 2.9-fold increase in the unbound fraction. The low binders bromfenac and indomethacin displayed only 1.5- and 1.3-fold differences in the free drug fraction between the assays, respectively. Melanin binding competition might involve several ocular and systemic drugs that may be concomitantly used, potentially leading to drug-drug interactions. Significance of such interactions depends on the extent of melanin binding, concentrations of the drugs in the pigmented cells and pharmacological responses of the involved compounds.

Earlier studies have shown that some drugs in our compound set bind to melanin in vivo. The accumulation of betaxolol in human and monkey pigmented ocular tissues was shown by Hollo et al (2006). In the human study, glaucoma patients with scheduled enucleation administered betaxolol eye drops 28 days prior the surgery (Hollo et al., 2006). The study showed that the pigmented tissues had the highest betaxolol concentrations: the highest concentrations were detected from iris, ciliary body and choroid. The results were similar in monkeys: after 30 days of topical betaxolol treatment, betaxolol concentrations were highest in iris-ciliary body and choroid. Likewise, topical administration of atropine and pilocarpine leads to melanin binding and longer duration of action in pigmented animals compared to albino animals (Salazar and Patil, 1976; Urtti et al., 1984). However, melanin binding can also hinder drug response: timolol response is weaker in pigmented than in albino animals (Nagata et al., 1993), and in humans with brown irides compared to humans with blue eye color 1 hour after installation (Salminen et al., 1985).
Interestingly, in this study pilocarpine and atropine were low and intermediate binders with 30.5% and 55.8% binding to melanin, respectively (Fig. 2, Table S1). In an earlier study, timolol was shown to bind to melanin more extensively than pilocarpine (binding percentages approximately 80% and 40%, respectively) (Nagata et al., 1993). These results indicate, that melanin binding of pilocarpine and atropine results in longer exposure of the receptors to the free drug and thus prolonged response. On contrary, exposure of beta-receptors to free timolol is decreased due to the increased pigment binding in brown eyes.

Treatment of posterior eye conditions such as retinopathies is challenging due to the difficulties in retinal drug delivery. Currently, intravitreal injection is the most common method to deliver drugs to the retina, and it is routinely used with antibodies that are injected monthly in the treatment of the wet form of AMD. However, small molecular weight compounds are not suitable for intravitreal injections due to their short elimination half-lives (a few hours) in the vitreous. Since pigment binding may occur without adverse effects (Leblanc et al., 1998), it represents an interesting option for prolonged drug action in the retina. This was recently demonstrated with a small molecular weight anti-VEGF agent in rodents, where orally administered panzopanib retained in pigmented ocular tissues at least for 35 days (Robbie et al., 2013).

For efficient retinal delivery after systemic administration, sufficient levels of free drug must be available in the blood stream (Vellonen et al., 2016). Thus, to target the compounds from systemic blood stream to pigmented ocular tissues, the compounds should bind to melanin at moderate levels, but not extensively to plasma proteins. We compared our melanin binding data to plasma protein binding (values collected from (Obach et al., 2008; Votano et al., 2006). Linear correlation between melanin binding and plasma protein binding of drugs was not observed. Dotted lines represent 50% and 80% of binding on both axes.

Figure 4. Plasma protein and melanin binding comparison of 28 compounds in our cassette analysis. Melanin binding values from 10 mg/ml melanin cassette assay were used. Plasma protein binding values were collected from earlier literature (Obach et al., 2008; Votano et al., 2006). Linear correlation between melanin binding and plasma protein binding of drugs was not observed. Dotted lines represent 50% and 80% of binding on both axes. There was no correlation between our melanin binding and plasma protein binding (Fig. 4) suggesting that these binding events are very different. If the aim is to target pigmented ocular tissues via systemic administration, compounds with intermediate or high melanin binding and low to intermediate plasma protein binding would be preferable. For instance, labetalol was seen to accumulate into uveal tract after oral dosing in dogs (Poynter et al., 1976). Labetalol displays 50% binding to plasma proteins (Obach et al., 2008; Votano et al., 2006). Comparison of melanin binding and plasma protein binding could be exploited in drug discovery as a lead compound selection criteria, when pigment binding targeting systems are developed.

Pigment binding in vivo is also affected by the drug transport across cellular and melanosomal membrane. However, only few studies describe pigment binding using isolated organelles, melanosomes (e.g. (Abrahamsson et al., 1988; Debing et al., 1988; POTTS, 1964b; Wilczok et al., 1990). and the quality of the isolated organelles used in these studies remains unclear, since functionality and intactness of the organelles were not determined. Furthermore, melanin binding has not been extensively studied at cellular level. Several transporter proteins are expressed on the RPE plasma membrane (Mannermaa et al., 2006; Pelkonen et al., 2017), and the efflux protein MRP4 is found at the melanosomal membrane (Azarian et al., 2006). Passive and active transport rates across cell and melanosomal membrane may have significant impact on the drug access to RPE melanosomes. According to simulations conducted in our group (Del Amo et al., 2016), melanin binding and cell membrane permeability have significant roles in the intracellular exposure to free drug in the RPE. Therefore, the extent of melanin binding needs to be considered in ocular pharmacokinetics.
In conclusion, we introduce a novel platform for melanin binding screening with commercially available RED inserts. Our results provide insights into melanin binding of different ocular and systemically used drugs and can further be exploited to predict pharmacokinetics in the RPE.

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References


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Fig. 1

1 Assays 1–4 cassette drug mix; Assay 5: individual compounds. Drug concentration of 500 ng/ml of each drug was used.
2 Melanin concentrations 1 mg/ml; 2 mg/ml; 10 mg/ml; 20 mg/ml in the Assays 1–5, respectively (final concentration in the wells 0.5–20 mg/ml)
Fig. 2
Melanin binding at equilibrium

different assay conditions

% Bound to melanin

Methotrexate
Methazolamide

Bromfenac

Indomethacin

Naloxone
Atropine
Tetracycline

Ciprofloxacin

↓ No binding

*Binding could not be determined

Fig. 3
Fig. 4
Graphical abstract