Original research article

Melanin targeting for intracellular drug delivery: Quantification of bound and free drug in retinal pigment epithelial cells

Anna-Kaisa Rimpelä[a], Marja Hagström[a], Heidi Kidron[a], Arto Urtti[a,b,c,*]

[a] Drug Research Program, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, P.O. Box 56, FI-00014 University of Helsinki, Finland
[b] School of Pharmacy, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland
[c] Institute of Chemistry, St. Petersburg State University, Petergof, St. Petersburg 198504, Russia

ARTICLE INFO

ABSTRACT

Melanin binding affects drug distribution and retention in pigmented ocular tissues, thereby affecting drug response, duration of activity and toxicity. Therefore, it is a promising possibility for drug targeting and controlled release in the pigmented cells and tissues. Intracellular unbound drug concentrations determine pharmacological and toxicological actions, but analyses of unbound *vs.* total drug concentrations in pigmented cells are lacking. We studied intracellular binding and cellular drug uptake in pigmented retinal pigment epithelial cells and in non-pigmented ARPE-19 cells with five model drugs (chloroquine, propranolol, timolol, diclofenac, methotrexate). The unbound drug fractions in pigmented cells were 0.00016–0.73 and in non-pigmented cells 0.017–1.0. Cellular uptake (i.e. distribution ratio Kp), ranged from 1.3 to 6300 in pigmented cells and from 1.0 to 25 in non-pigmented cells. Values for intracellular bioavailability, F_{ic}, were similar in both cells types (although larger variation in pigmented cells). *In vitro* melanin binding parameters were used to predict intracellular unbound drug fraction and cell uptake. Comparison of predictions with experimental data indicates that other factors (e.g. ion-trapping, lipophilicity-related binding to other cell components) also play a role. Melanin binding is a major factor that leads to cellular uptake and unbound drug fractions of a range of 3–4 orders of magnitude indicating that large reservoirs of melanin bound drug can be generated in the cells. Understanding melanin binding has important implications on retinal drug targeting, efficacy and toxicity.

1. Introduction

Drug-melanin binding is known to accumulate drugs into pigmented tissues, especially in the eye [1–4]. The eye is the most densely pigmented organ in the body [5], and melanin can be found in the anterior segment in the iris and ciliary body, and in the posterior segment in the choroid and retinal pigment epithelium (RPE). Melanin binding is a prevalent phenomenon, as various clinical drugs bind to melanin to some extent [6]. In the case of ocular drugs, binding has been shown to modulate drug distribution to the pigmented tissues [7, 8] as well as the drug response [9]. Systemically administered drugs can also accumulate to the pigmented tissues of the eye, a phenomenon demonstrated most extensively with chloroquine [10, 11]. Therefore, high-binding drugs can have ocular effects even though the therapeutic target of the drug is not in the eye. Melanin binding is an important factor to be considered in ocular as well as other drug therapy and development.

Drug retention in the pigmented tissues has regularly been shown as elevated total drug concentrations in these tissues, compared to the corresponding levels in the albino tissues [8, 10–12]. The free, pharmacologically effective concentrations, especially in the case of high-binding drugs, may be much lower than the total concentrations [4]. The total concentrations, therefore, are poor indicators for pharmacodynamic drug responses in these tissues. Melanin binding has been connected, somewhat misleadingly, to drug toxicity in pigmented tissues. Although the binding can accumulate drugs to these tissues, in fact the free, and not the total concentration in the cells, determines the toxicity. Melanin can, therefore, also protect the cells from the harmful drug reactions. Drug toxicity in the pigmented cells depends on the pharmacodynamic properties of the drug, and it has been shown that melanin binding itself does not predict ocular toxicity [3].

Although attention has been paid to the unbound drug fractions in non-pigmented cells and their relation to drug action [13–16], unbound fractions in pigmented cells have not been investigated. As the differences between the total and free concentration can be expected to be higher in pigmented than in non-pigmented cells, it is of high importance to relate the total concentration to the free concentration in

---

pigmented cells. Melanin binding has been implied as a possible drug targeting approach, to sustain the action of drugs in pigmented ocular tissues by a melanin-bound drug depot [12]. To be able to materialize this approach, it would be of great benefit to understand the relationship of the total and unbound (effective) concentration in these tissues based on the extent of *in vitro* melanin binding of targeting drug candidates.

We investigated the unbound fraction and the cellular uptake of five model drugs with varying melanin binding as well as physicochemical properties, with pigmented primary RPE cells and a non-pigmented RPE cell line (ARPE-19), to demonstrate the effect of drug-melanin binding on the intracellular unbound drug fraction as well as cell uptake in pigmented cells. We also predicted the unbound fractions and cell uptake with melanin binding parameters to bridge the gap between *in vitro* binding to isolated melanin and cell level drug retention.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO) and all of the drug compounds, chloroquine diphosphate salt, diclofenac sodium salt, methotrexate hydrate, propranolol hydrochloride and timolol maleate salt, were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMSO was used as a solvent for the highest concentrations of stock solutions (50 mM) of diclofenac, methotrexate (also 5 mM) and timolol. The other stock solutions were made in Milli-Q water. Dilutions were made in the buffer of choice for the study, either in Hank's balanced salt solution (HBSS, with $CaCl_2$, $MgCl_2$) or Dulbecco's phosphate buffered saline (DPBS, without $CaCl_2$, $MgCl_2$) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Methods

2.2.1. Isolation of primary RPE cells

The isolation of primary porcine RPE (pRPE) cells was done as previously reported [17]. Briefly, the extraocular tissues and the anterior part of the eye were removed and the vitreous poured out of the eye. The eye was filled with DPBS (without $CaCl_2$, $MgCl_2$) and incubated for 10 min. The neural retina was carefully removed, the eyecup was filled with 0.25% trypsin-EDTA (Gibco, Thermo Fisher Scientific) and incubated at 37 °C for 30–35 min. The detached cells were collected and twice the volume of the cell suspension of growth medium (10% (v/v) fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin in DMEM 31885 (Gibco, Thermo Fisher Scientific)) was mixed with the cells. The cells were pelleted three times (once at 450 g for 5 min and twice at 200 g for 2 min), replacing the supernatant with fresh growth medium in between and at the end. The cells were then transferred to a T25 cell culture flask (Sarstedt, Nümbrecht, Germany). Twenty eyes were used for one flask.

2.2.2. Cell culture

ARPE-19 cells (ATCC, Manassas, VA, USA) were cultured in T75 or T175 cell culture flasks at 37 °C (humidified atmosphere, 7% CO_2) using DMEM/F-12 (Gibco, Thermo Fisher Scientific), supplemented with 10% (v/v) fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin, as growth medium. The cells were subcultured once a week and the growth medium changed once in between. Cell passages 13–26 were used for the studies.

The pRPE cells were grown in the T25 flask for 6–8 days before the experiments, at 37 °C (humidified atmosphere, 5% CO_2). The medium was changed the day after isolation, and once more before collecting the cells for experiments.

2.2.3. Intracellular drug binding

For studying intracellular binding of the drugs, a previously described method [13, 18] was used with small modifications. The ARPE-

19 cells were collected with TrypLE™ Express (Gibco, Thermo Fisher Scientific) and the pRPE cells with 0.25% trypsin-EDTA. The cells were then washed once with DPBS (without $CaCl_2$, $MgCl_2$), counted in a Bürker chamber, and centrifuged to remove the supernatant. The cell pellet was stored at −20 °C. Before the experiment, the pellet was thawed, the cells suspended in HBSS at a density of 10×10^6 cells/ml for ARPE-19 cells and 1×10^6 cells/ml for pRPE cells, and the cell suspension homogenized with a tip sonicator (Vibra-Cell Ultrasonic Liquid Processor VCX500, Sonics, Newtown, CT, USA) for 10 s at 20% intensity.

The binding was studied in triplicates in rapid equilibrium dialysis (RED) inserts (Thermo Scientific) with 190 μl of the cell homogenate, spiked with 10 μl of the compound, in the donor chamber (homogenate chamber) and 350 μl of HBSS in the receiver chamber (buffer chamber). Each drug was studied at concentrations 0.5 and 5 μM. The inserts were incubated at 37 °C with 900-rpm shaking for 5–6 h, depending on the equilibrium time of each compound (methotrexate 6 h, others 5 h). To assess the mass balance of the experiment and compound stability, RED insert controls were used with compound spiked buffer (instead of cell homogenate) in the donor chamber and well plate controls without the RED insert with 200 μl of either compound spiked cell homogenate or compound spiked buffer and 350 μl of HBSS added to the same well. The mass balance showed recovery values over 88% on all occasions.

After the incubation time, a 40-μl sample was taken from both chambers, and 40 μl of cell homogenate or HBSS was added to the buffer and homogenate samples, respectively, to give identical matrices. To these samples, 80 μl of acetonitrile (ACN), 0.2% formic acid was added to release the drug and precipitate the protein. The samples were then centrifuged at 15000g for 20 min. The supernatant was collected for analysis, the samples from the 5 μM experiments were further diluted 1:10 with 50:50 [HBSS: (ACN, 0.2% formic acid)]. Internal standards were added (more in 2.2.7 Analytical methods) to the samples and the samples stored at −80 °C until analysis.

The unbound fraction inside the cells ($f_{u,cell}$) was calculated as presented by Mateus et al. [13] with Eqs. 1 and 2

$$f_{u,hom} = \frac{C_{buffer}}{C_{hom}} \tag{1}$$

$$f_{u,cell} = \frac{1}{D*(1/f_{u,hom} - 1) + 1} \tag{2}$$

where $f_{u,hom}$ is the unbound fraction in the homogenate, C_{buffer} is the concentration in the buffer chamber and C_{hom} in the homogenate chamber. D is the dilution factor taking into account the dilution caused by the *in vitro* experiment. D was estimated to be 73 for ARPE-19 cells (10×10^6 cells/ml, cell diameter 13.8 ± 0.6 μm) and 1100 for pRPE cells (1×10^6 cells/ml, cell diameter 12.0 ± 0.2 μm) assuming a spherical shape for the cells. Cell diameters were measured with Cedex XS Cell Analyzer (Roche CustomBiotech, Penzberg, Germany).

2.2.4. Cell uptake and intracellular bioavailability

On the day before the cell uptake experiment, the cells were seeded on 48-well plates (Nunclon Delta surface, 1.1 cm²/well, Thermo Scientific) at a density of 100,000 cells/well as described previously [17]. TrypLE™ Express was used with ARPE-19 cells instead of 0.25% trypsin-EDTA, and the growth media were the same as mentioned earlier in 2.2.2 Cell culture. The cells were grown in the wells for 20–24 h before the experiment.

Prior to the experiment, the cells were washed twice with HBSS. They were then incubated with 250 μl of the drug in HBSS at 37 °C with 175-rpm shaking for 5 h. All drugs were studied in triplicates at the concentrations 0.5 and 5 μM. After the incubation, the medium (drug solution above the cells in HBSS) was collected for analysis. The cells were then washed twice with ice-cold DPBS to remove the drug not associated with the cells. Subsequently, the cells were frozen at −80 °C for 2 h, thawed at room temperature for 30 min, and lysed with 100 μl

of Milli-Q water for 10 min, and additional 15 min with 150 μl of ACN, 0.2% formic acid (together with the Milli-Q water) to release the drug associated with the cells. The lysate was then collected and centrifuged at 20000*g* for 20 min. The supernatant was collected for analysis and internal standards added before storing the samples at −80 °C until analysis. The 5 μM samples were diluted 1:10 with 40:60 [Milli-Q water: (ACN, 0.2% formic acid)] prior to the addition of the internal standards.

The distribution ratio (*Kp*) describing the cell uptake was calculated according to Eq. 3 assuming a cell volume (*V*cell) of 1 μl.

$$Kp = \frac{A_{cell}/V_{cell}}{C_{medium}} \tag{3}$$

where A_{cell} is the amount in the cell lysate and C_{medium} is the concentration measured from the medium above the cells.

The intracellular bioavailability (F_{ic}), previously determined by Mateus et al. [13, 18], was calculated with Eq. 4

$$F_{ic} = f_{u,cell} * Kp \tag{4}$$

2.2.5. Determining the melanin content in the cells

Cellular melanin content in the intracellular drug binding and cell uptake experiments was determined by spectrophotometry, to ensure a consistent pigmentation throughout the study and to use the melanin content values in the predictions of cell uptake and unbound drug fractions. Equal volumes of cell homogenate and 20% DMSO in 2 M NaOH were mixed. The suspension was then heated at 70 °C for 1 h, sonicated for 10 min and the absorbance measured at 475 nm (Varioskan LUX™ Multimode microplate reader, Thermo Fisher Scientific). In the cell uptake experiment, the cells on the 48-well plate were washed twice with DPBS, subsequently adding 100 μl of Milli-Q water and 150 μl of 20% DMSO in 2 M NaOH in the well and incubating for 15 min. The lysed cells were collected from the well and treated similarly as the homogenate above.

Isolated porcine ocular melanin from the RPE-choroid (isolation method previously described [17]) was used as the reference to quantitate the melanin content. A 1-mg/ml stock solution of melanin in DPBS was prepared and treated as described for the cell homogenate above. It was then diluted to multiple concentrations for the standard curve. Melanin content of the non-pigmented ARPE-19 cells was also measured in the same manner, but the absorbance was negligible, and therefore, the standard curve could be made of melanin only, without cell material.

2.2.6. Melanin binding studies

Melanin binding of diclofenac and propranolol was determined for calculating the binding parameters, maximum binding capacity (B_{max}), dissociation constant (K_d), and heterogeneity index (*n*) for the prediction of cell uptake and intracellular unbound fraction. Melanin binding was studied with isolated porcine RPE-choroidal melanin with a previously described method [17] with concentrations 0.5–500 μM of the drug and 1 mg/ml melanin, in DPBS (pH 7.4). For chloroquine, timolol, and methotrexate, previously reported binding parameters [17] (pH 7.4) were used. The Sips isotherm was used for the analysis of the binding parameters [17, 19]. The curve fitting was done in Phoenix WinNonlin (Certara, Princeton, NJ, USA) with $1/Y^2$ weighting for propranolol.

2.2.7. Analytical methods

Samples from the intracellular drug binding and cell uptake experiments were analyzed with an LC-MS/MS technique. Liquid chromatography (LC) separations were carried out using Waters Acquity UPLC (Waters, Massachusetts, USA), Waters Acquity HSS T3 (1.8 μm, 2.1 × 100 mm) column and gradient elution. The solvents used were 0.1% of ACS grade formic acid (Merck, Darmstadt, Germany) in ultrapure water (A) and 100% of LC-MS grade ACN (B). More detailed

information of the gradients is presented in Supplementary Table 1. The flow rate was 0.4 ml/min and the injection volume varied from 0.1 to 1 μl depending of the compound. The column temperature was 26 °C. For chloroquine, propranolol and timolol, isopropanol injections were run between every sample to prevent carry-over. With other compounds, blank samples were injected after every third sample to accomplish the same.

Mass spectrometric (MS) measurements were carried out using Waters Xevo triple quadrupole mass spectrometer (TQ-S) equipped with a Waters electrospray ionization (ESI) source operated in positive ion mode. The optimal source parameters were compound dependent and varying; capillary voltage from 2.1 to 3.5 kV, cone voltage from 2 to 45 V, desolvation temperature from 500 to 600 °C and desolvation gas flow from 900 to 1000 L/h. Source temperature was the same in all methods, 150 °C, as was the cone gas flow, 150 l/h, and collision gas flow, 0.15 ml/min. Nitrogen was used as the desolvation, nebulizer and cone gases and argon as the collision gas. Multiple reaction monitoring (MRM) was used as the scan type. The detailed parameters for each compound can be found in Supplementary Table 2. The internal standards were as follows: propranolol for chloroquine and timolol, atenolol for methotrexate, prednisolone for propranolol, and indomethacin for diclofenac. Internal standard concentrations in the final sample and standard solutions ranged from 10 to 100 nM. The resulting data were analyzed with Waters MassLynx software.

Samples from the melanin binding experiments (propranolol and diclofenac) were analyzed with UPLC (Acquity UPLC, Waters, Milford, MA, USA) combined with UV detection (Photodiode Array Detector, Waters, USA). The separation was carried out on an Ascentis Express RP-amide (2 μm, 2.1 × 50 mm) column (Supelco, Sigma-Aldrich, Bellefonte, PA, USA) at 30 °C with an injection volume of 2.5 μl and flow rate of 0.5 ml/min. Both compounds were run on an isocratic mode with a mobile phase consisting of 15 mM phosphate buffer (A) and ACN (B) (80% A/20% B for propranolol, 45% A/55% B for diclofenac).

The analytical methods were qualified for the purpose. The lower limits of quantification (LLOQ) for the LC-MS/MS method were as follows: chloroquine, timolol and propranolol 0.5 nM, methotrexate 1 nM, and diclofenac 5 nM. The LLOQs in the UPLC method were 0.1 μM for propranolol and 0.5 μM for diclofenac. Limit of quantification (LOQ) was considered at a signal-to-noise ratio of 9:1.

2.2.8. Predicting intracellular drug binding, cell uptake and intracellular bioavailability

Cellular uptake and unbound fraction inside the cells were calculated based on the melanin binding parameters B_{max}, K_d, and *n*. The fraction inside the cells (f_{pred}) was calculated as reported in [17] with Eq. 5, and the unbound fraction inside the cell ($f_{u,cell,pred}$) with Eqs. 6, 7 and 8.

$$m = \left(1 + \frac{K_d^n}{([L]_0(1-f_{pred}))^n}\right)$$
$$* \frac{[L]_0 V_w - [L]_0(1-f_{pred})(V_w + V_{cell})}{B_{max}} \tag{5}$$

$$[L] = [L]_0(1-f_{pred}) \tag{6}$$

$$B_{cell} = m_{cell} * \frac{B_{max}[L]^n}{K_d^n + [L]^n} \tag{7}$$

$$f_{u,cell,pred} = \frac{A_{free,cell}}{A_{total,cell}} = \frac{[L]V_{cell}}{[L]V_{cell} + B_{cell}} \tag{8}$$

where *m* is the amount of melanin (mg), [L] is the free drug concentration (nmol/ml = μM), $[L]_0$ is the original drug concentration in the medium (μM), V_w is the volume of the medium in the well (0.25 ml), V_{cell} is the volume of the cells (ml) (assumed to be 1 μl = 0.001 ml), B_{cell} is the bound drug amount inside the cells (nmol),

Table 1
The acid/base status, pK_a and $logD_{7.4}$ values for the studied drugs (ACD predicted [21]).

Drug	$pK_{a,base}$	$pK_{a,acid}$	$logD_{7.4}$
Chloroquine	10.4	–	1.6
Propranolol	9.5	–	0.8
Timolol	9.4	–	−0.6
Diclofenac	–	4.2	1.4
Methotrexate	–	3.5	−5.1

and m_{cell} is the specific measured amount of melanin inside the cells (mg).

Kp values were predicted based on melanin binding ($Kp_{pred,mel}$) with Eq. 9

$$
\begin{aligned}
Kp_{pred,mel} &= \frac{A_{total,cell} / V_{cell}}{A_w / V_w} \\
&= \frac{A_{total,cell} V_w}{A_w V_{cell}} \\
&= 250 * \frac{f_{pred}}{(1 - f_{pred})}
\end{aligned}
\tag{9}
$$

where the ratio $A_{total,cell}/A_w$ (the amount of drug in the medium) can be expressed as the ratio of the predicted fractions of drug inside (f_{pred}) and outside (1-f_{pred}) the cells. The ratio V_w/V_{cell} equals to 250.

F_{ic} values were predicted ($F_{ic,pred}$) based on the pH partitioning theory as presented in [13]. The pK_a values presented in Table 1 were used, and the pH values assumed for melanosomes and lysosomes were 5.0, cytosol 7.1, and medium outside the cells 7.4. The volume of lysosomes was assumed to be 1% of the total cell volume and the volume of melanosomes 7% of the cell volume [20].

Based on $F_{ic,pred}$, Kp values were predicted (Kp_{pred}) with Eq. 10, taking into account both the melanin binding (in $f_{u,cell,pred}$) and pH partitioning in the subcellular compartments (in $F_{ic,pred}$).

$$
Kp_{pred} = F_{ic,pred}/f_{u,cell,pred}
\tag{10}
$$

3. Results

3.1. Melanin binding

Melanin binding was studied with propranolol and diclofenac (Table 2). Melanin binding of diclofenac could not be detected in the experimental conditions (0.5–500 μM drug, 1 mg/ml melanin, pH 7.4) and the binding parameters could not be calculated. Propranolol binding parameters are reported in Table 2. Melanin binding of the other drugs, chloroquine, timolol and methotrexate, has been studied previously [17] and these parameters were used here (Table 2). From here on, chloroquine, propranolol, and timolol, will be called melanin binders and diclofenac and methotrexate low/non-binders (although their binding was not detectable in the conditions of this study, they have low binding levels in other conditions [6, 17]).

3.2. Intracellular drug binding, cell uptake and intracellular bioavailability

The intracellular unbound drug fraction ranged from 0.00016 to

0.73 in the pigmented pRPE cells and from 0.017 to 1.0 in the non-pigmented ARPE-19 cells (Fig. 1A). It was clear that melanin binding decreased the unbound fraction, as the melanin-binding drugs, chloroquine, propranolol, and timolol, had 10–300-fold lower unbound fractions in pigmented cells, whereas the low/non-binders, methotrexate and diclofenac, showed 0–10-fold lower unbound fractions.

Cell uptake values showed clear differences between drugs and pigmented *vs.* non-pigmented cells: Kp was 1.3–6300 in pRPE cells and 1.0–25 in ARPE-19 cells (Fig. 1B). Melanin binders had again much higher uptake to the pigmented than the non-pigmented cells, with 30–270-fold differences. The uptake of the low/non-binders was similar in both cell types. Chloroquine uptake was much lower than expected, based on the extent of melanin binding, as well as our previous cell uptake results of chloroquine [17]. Possible reasons for this are discussed later.

The F_{ic} values ranged from 0.28–2.2 in the non-pigmented cells and from 0.04 to 8.2 in the pigmented cells (Fig. 1C). In theory, the intracellular bioavailability (F_{ic}) is expected to be 1 for passively diffusing drugs and above 1 for positively charged drugs that get trapped in acidic intracellular compartments (ion trapping) such as lysosomes and melanosomes. Melanin binding, or other cellular binding, should not affect the F_{ic}, as the extent of binding-related cell uptake is compensated by the lowering of the unbound fraction. On average, the F_{ic} was lower for the acidic compounds than the basic compounds (Table 3), as can be expected due to ion trapping.

3.3. Predicted intracellular unbound fraction, cell uptake and intracellular bioavailability

The unbound drug fractions inside the pigmented cells were predicted based on the melanin binding parameters in Table 2. The measured melanin amount inside the cells, 25.4 ± 1.2 μg/well (mean ± SD), was used in the predictions. The predicted unbound fractions of the melanin binders were approximately 10-fold higher than the measured values (Fig. 2A). For the low/non-binding drugs, the predicted value was 1 (as they do not bind to melanin, which is the only predicting factor in this case). This was close to the measured value for methotrexate (0.7) but 100-fold higher than the measured value for diclofenac (0.01). As other cellular binding also affects the unbound fraction, the lower measured unbound fraction of diclofenac can be explained by its lipophilicity. The correlation coefficient of the predicted and measured values was 0.73 (log-log plot) (Supplementary Fig. 1), and it is clear that lipophilicity and binding to other cell components, especially in the case of low/non-melanin-binding drugs, should be accounted for when assessing the unbound fraction.

Cell uptake to the pigmented cells was also predicted with the melanin binding parameters. The predicted uptake ($Kp_{pred,mel}$) was 10–100-fold lower than the measured values for the basic drugs, propranolol and timolol (melanin binders) (Fig. 2B). Chloroquine was an outlier here (because of the measured low cell uptake), and the predicted value was similar to the measured value. The predicted value should be lower than the measured value for all basic drugs, as pH partitioning to the subcellular acidic compartments (melanosomes, lysosomes) as well as other cellular binding increase the uptake. For the acidic low/non-binders, the melanin-binding-predicted Kp values

Table 2
Melanin binding parameters (mean ± SEM) for the studied drugs from left to right in the decreasing order of melanin binding.

Parameter	Chloroquine[a]	Propranolol	Timolol[a]	Diclofenac	Methotrexate[a]
B_{max} (nmol/mg)	380 ± 40	176 ± 60	39 ± 6	–	–
K_d (μM)	76 ± 23	163 ± 100	120 ± 30	–	–
n	0.61 ± 0.01	0.89 ± 0.05	0.95 ± 0.03	–	–
% bound at 1 μM	99.6	70	32	0	0

[a] [17]
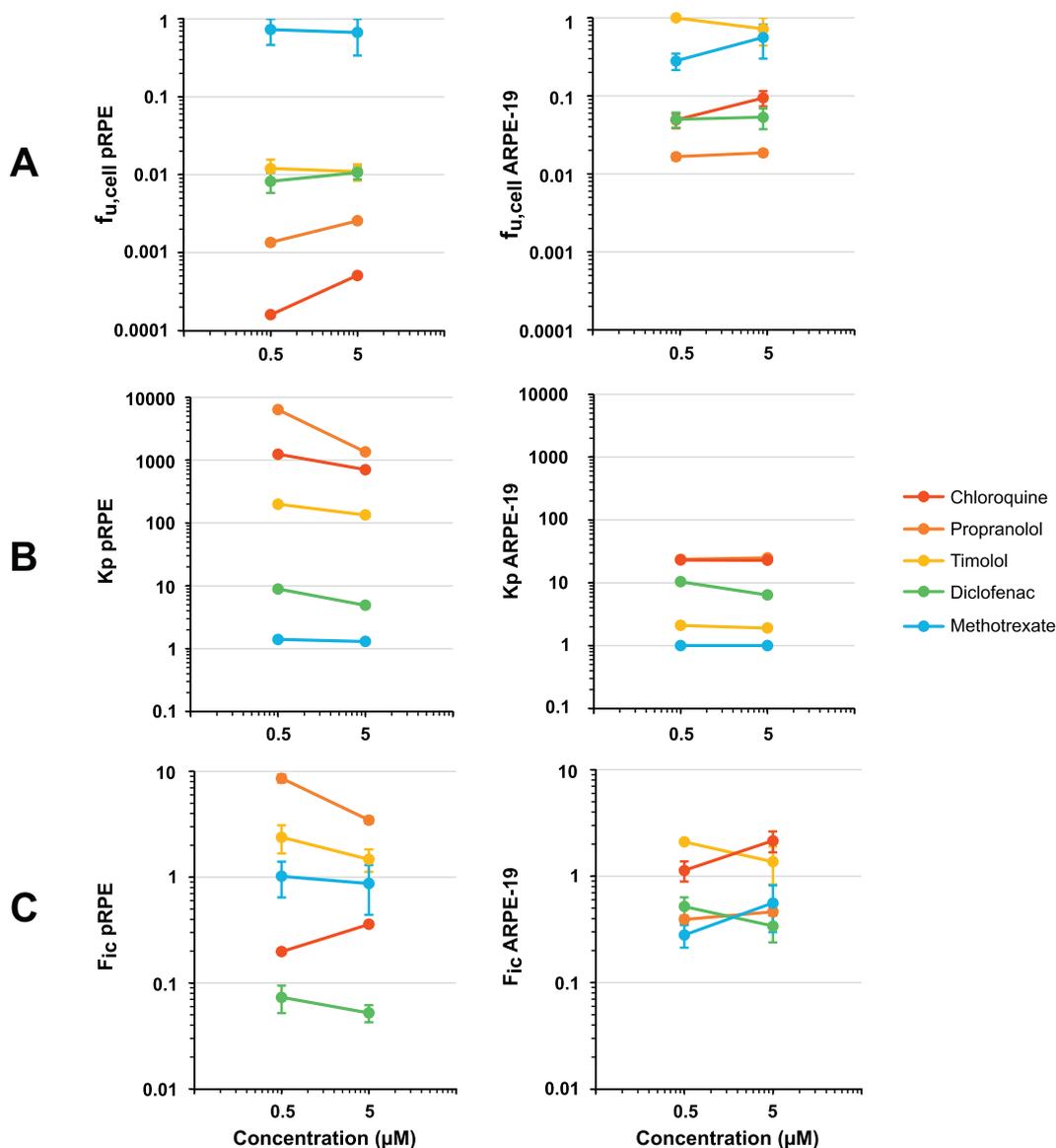
Fig. 1. Measured values in pRPE (left) and ARPE-19 cells (right), A: intracellular unbound fractions ($f_{u,cell}$), B: cell uptake (Kp), C: intracellular bioavailability (F_{ic}). The error bars describe the standard error of mean (SEM), and are in some cases encompassed by the symbol.

Table 3
Predicted and measured F_{ic} (mean $\pm$ SEM) grouped by the acid/base status of the drug.

		$F_{ic,pred}$	$F_{ic,measured}$
Basic	pRPE	22 $\pm$ 0.1	2.7 $\pm$ 1.2
	ARPE-19	4.5 $\pm$ 0.02	1.3 $\pm$ 0.3
Acidic	pRPE	0.46[a]	0.50 $\pm$ 0.3[b]
	ARPE-19	0.50[a]	0.44 $\pm$ 0.1[b]

[a] Mean and SEM not calculated, as there were only two data points for the predicted values. Both data points had values rounding up to the presented value.

[b] Mean and SEM calculated of the individual four data points (two drugs with two concentrations).

compared to the measured values similarly as in predicting the unbound fraction. $Kp_{pred,mel}$ for methotrexate was similar than the measured Kp, but for diclofenac, the predicted value was 10-fold lower. This, again, can be explained by the lipophilicity of the drug causing binding to other cellular components than melanin. The correlation coefficient between the measured and melanin-binding-predicted

values (0.5 μM study concentration) on a log-log plot was 0.64 (Supplementary Fig. 2). Cell uptake was also predicted taking into account the pH partitioning (Fig. 3) in the subcellular compartments in addition to melanin binding (Kp_{pred}) (Fig. 2B). This improved the log-log correlation to the measured values from 0.64 to 0.79 (Supplementary Fig. 2).

The F_{ic} values in both cell types were predicted according to pH partitioning to different subcellular compartments (cytosol, lysosomes, melanosomes) (Fig. 3) based on the pK$_a$ values of the drugs studied (Table 1). The measured values for the basic drugs were lower in both cells than the predicted values, but for the acidic drugs the measured and the predicted values were similar (Table 3). The differences in the measured and predicted values were comparable to a previous study [13], where a much wider group of drugs was studied with non-pigmented cells. The difference in the predicted values in different cell types arises from the volume difference of the pH 5.0 compartments (only lysosomes in ARPE-19 cells *vs.* lysosomes and melanosomes in pRPE cells).
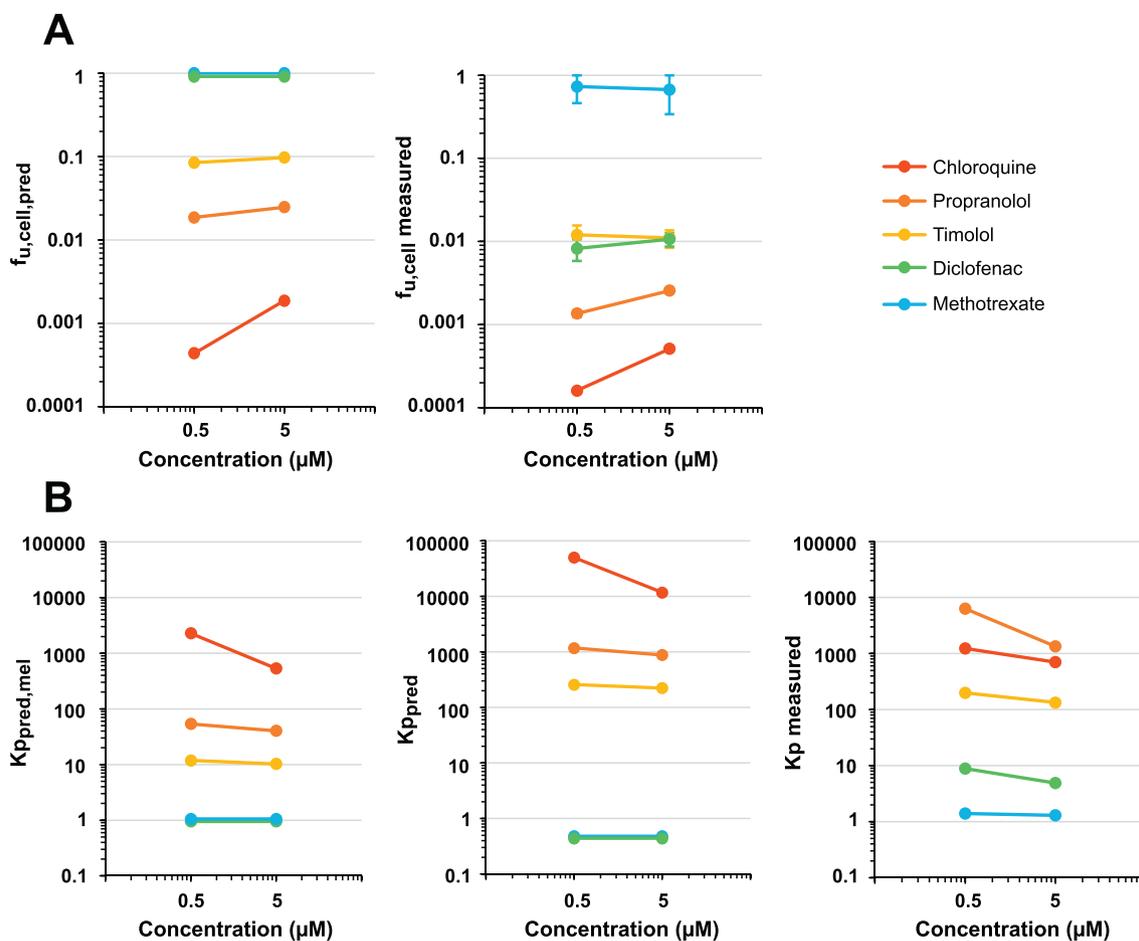
Fig. 2. Predicted *vs.* measured values, A: intracellular unbound fractions ($f_{u,cell,pred}$), B: cell uptake ($Kp_{pred,mel}$ and Kp_{pred}). The error bars describe SEM (there are no error bars in the predicted values).
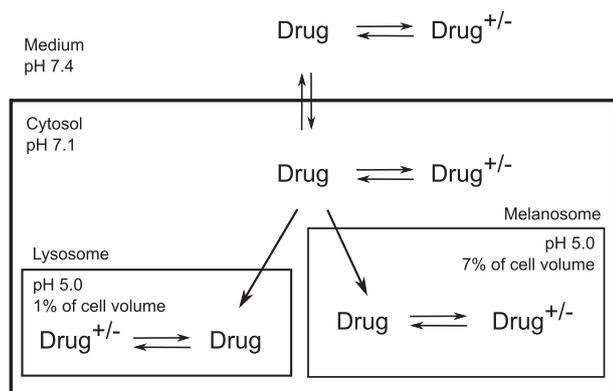
Fig. 3. pH partitioning inside the pigmented and non-pigmented cells. Non-pigmented cells were assumed not to have melanosomes.

4. Discussion

To obtain targeted and sustained drug action through melanin binding, a key aspect is the capability of the free concentration to produce a sustained drug response without causing toxicity. As intracellular unbound drug fraction represents the effective fraction of the drug inside the cells, it is a more important factor than the total concentration in understanding the pharmacodynamic effect of the drug. We measured intracellular unbound drug fractions and cell uptake of five model drugs with different melanin binding (Table 2) and physicochemical (Table 1) properties comparing pigmented and non-

pigmented RPE cells.

The intracellular unbound fractions were lower in pigmented than non-pigmented cells for all of the drugs except for methotrexate. Methotrexate had similar unbound fraction, close to 1, in both cell types, as can be expected since it is a low- or non-melanin-binding, hydrophilic drug. The unbound fraction of diclofenac, also a low/non-binding drug, was up to 10-fold lower, and the unbound fractions of the melanin-binding drugs, timolol, propranolol, and chloroquine, 10–300-fold lower in pigmented than non-pigmented cells. The lowering of the unbound fraction did not directly correlate with the extent of *in vitro* melanin binding (isolated porcine RPE-choroidal melanin), but due to the interplay of other cellular binding together with melanin binding and the low number of compounds studied, it is difficult to obtain comprehensive correlations and specify reasons. Nevertheless, the rank order of the unbound fractions was in the order of melanin binding, except for the lipophilic diclofenac which had similar unbound fraction than the moderately melanin-binding timolol. Therefore, it can be concluded that melanin binding plays an important role in determining the unbound fraction, but other cellular binding events also contribute.

The cell uptake of the melanin-binding drugs was much higher (30–270-fold) in pigmented than non-pigmented cells. The uptake of the low/non-binders was similar in both cells, thus, correlating with the result of low/non-existent *in vitro* melanin binding. Therefore, we can say that melanin binding increased the drug uptake considerably. Surprisingly, propranolol had the highest uptake in the pigmented cells. Chloroquine, the highest melanin binder, would have been expected to have higher uptake than propranolol, due to the extensive melanin binding, as they had similar uptake in the non-pigmented cells. In our

previous experiments [17], chloroquine had much higher uptake in primary cells from the same source as in this study (Kp ~1000 here *vs.* 25,000 in [17]), adding to the unexpectedness of the low uptake here. The uptake of timolol, however, was similar in both studies (here and in [17]), therefore, other than melanin binding effects can be debated to cause the incoherence of chloroquine results. As an example, chloroquine is an MRP-1 efflux transporter substrate [22, 23]. RPE cells have been determined to express efflux transporters, such as MRP-1, -4, and -5 [24], and a high expression of the right efflux transporter could explain the differences. Timolol is a P-gp substrate but has not been reported to be a substrate for the above MRPs, therefore, allowing for different efflux outcomes for chloroquine and timolol. Other factors, such as differences in intramelanosomal pH (which is not known), can also participate in the differences. Chloroquine is, for example, known to increase the pH of lysosomes. A similar pH change may take place in melanosomes, thereby affecting the pH partitioning of chloroquine and causing variation in drug uptake. Nevertheless, the correlation between measured and predicted uptake values was improved (Supplementary Fig. 2, more information later) when chloroquine uptake results from this study were replaced with the higher uptake results from [17]. Therefore, it is safe to say that chloroquine uptake was an outlier in this study with a much lower than expected uptake into pigmented cells, as for the other drugs, melanin binding could explain the extent of uptake. It is clear that more drugs need to be studied to make more reliable conclusions and to reduce the effect of outliers on the outcome.

The unbound fraction and cell uptake in the non-pigmented ARPE-19 cells were similar as reported before with non-pigmented HEK293 cells for diclofenac and propranolol (compounds for which literature references could be found) [13]. Mateus et al. [18] have reported 10-fold lower unbound fractions for these drugs in MDCK cells (non-pigmented) than in HEK293 cells, corresponding with our result for the low/non-melanin-binder diclofenac in pigmented pRPE cells. As reported before by Mateus et al. [18] and others [25], the binding to non-pigmented cellular components is not dependent on the cell type, and is mainly governed by membrane partitioning of the compound. Although individual compounds may have some differences in different cell types, a correlation of unbound fractions in different cell types with a larger set of compounds has been seen. Our results are in accordance with this finding, as no higher than 10-fold differences were found for the low/non-melanin binders in the two cell types studied here.

We predicted the intracellular unbound drug fractions based on melanin binding parameters from *in vitro* binding studies. The predicted fractions were 10-fold higher than the measured values for the melanin-binding drugs. Of the low/non-binders, methotrexate had similar predicted and measured unbound fractions, but the predicted unbound fraction of diclofenac was 100-fold higher than the measured value. As other cellular binding than melanin binding affects the unbound fraction, it is clear that the whole unbound fraction cannot be predicted with only melanin binding (correlation on a log-log plot 0.73). Melanin binding could, however, predict the rank order of the melanin-binding drugs, and all the drugs except for diclofenac, and is a relevant predictor of the quantity of the unbound fraction. Nevertheless, experiments with more compounds are needed to make more detailed conclusions and correlations of the unbound fraction in pigmented cells with melanin binding as well as other parameters of the drug, such as lipophilicity, affecting the binding to other, non-melanosomal cell components.

Cell uptake was predicted with the melanin binding parameters and, additionally, considering both melanin binding and intracellular pH partitioning of the drug. Melanin binding explained the uptake partly (log-log plot correlation coefficient 0.64), but adding pH partitioning to the model, improved the correlation (0.79). Interestingly, when replacing the chloroquine uptake results of this study (outlier) with the much higher uptake results from our previous study [17] with a similar study setting, a clear improvement in the correlation of the predicted values was seen (Supplementary Fig. 3). The melanin-binding predicted cell

uptake ($Kp_{pred,mel}$) correlation with the measured Kp values improved from 0.64 to 0.88 and the melanin-binding and pH partitioning predicted cell uptake (Kp_{pred}) correlation improved from 0.79 to 0.94. Positively charged drugs tend to get trapped inside the acidic intracellular compartments, as the charged form does not penetrate the membranes as well as the neutral form. If melanosomes have an acidic intracellular pH, they increase the intracellular acidic compartment volume compared to non-pigmented cells that do not have melanosomes. The estimated area that melanin occupies in the RPE cell has been evaluated to be 7% [20]. Detailed information of melanosomal volume excluding the volume of melanin and information of lysosomal volumes of different cells is needed to assess the effect more precisely. Here, we assumed a melanosomal volume of 7% of the total cell volume for the pigmented cells and a lysosomal volume of 1% of the cell volume for both cell types. These volume differences caused a 4-fold higher predicted intracellular bioavailability for the basic drugs in pigmented, melanosome containing cells (Table 3). Therefore, ion trapping into melanosomes can increase the uptake to pigmented cells of even low-binding basic drugs compared to the uptake to non-pigmented cells. All in all, however, many factors affect this relationship and more information is required for a thorough understanding of intracellular distribution with and without melanin binding.

Although a convenient method to measure unbound intracellular drug fractions, the method used here does not take into account all the physiological factors regarding melanin binding. Melanosomal pH may play a role in the binding of drugs, a factor that cannot be considered with this method. Methotrexate, as an example, has been found to bind to melanin at pH values 4.8 and 5.0, although it does not bind at pH 7.4 [17, 26]. In the experimental conditions of this study, the melanin is expected to be in the pH environment of the *in vitro* setting (pH 7.4), as the homogenization method is expected to break the melanosomal membrane. Therefore, melanin binding of methotrexate is not expected to take place in these conditions. The pH inside melanosomes can be expected to be less than 7.4, but the exact pH of ocular melanosomes has not been determined [4]. Without a measured value for the intramelanosomal pH, the correct pH at which to study melanin binding *in vitro*, remains unknown. Therefore, definite conclusions cannot be made of the importance of *in vitro* melanin binding of methotrexate, or other compounds, at an acidic pH. Taking intramelanosomal pH into account is difficult with any other method than studying live whole cells. Estimation of the pH from isolated melanosomes, for example, is problematic, as fluorescent probes are generally used for pH determination, and melanin absorbs light of a wide range of wave lengths. Determining the unbound fractions from whole cells, on the other hand, is relatively challenging due to the requirement of separating the free and bound drug. Therefore, the cell homogenate binding used here is a relevant method. The benefit of this method is that it takes into account other cellular binding together with melanin binding, therefore, giving much better estimates of the unbound fraction inside the cells than *in vitro* studies with melanin alone.

As the isolation of primary RPE cells is somewhat laborious, a better source for melanin-containing cells with reproducible melanin levels comparable to *in vivo* tissue melanin content, would improve the significance of the method used here. Adult primary RPE cells do not synthesize melanin, and the melanin concentration is, therefore, diluted during cell division. Melanin-loaded ARPE-19 cells [27], melanin-synthesizing primary human fetal RPE cells [28] or highly pigmented stem-cell derived RPE cells [29] could be viable options to be used with this method, but have not been characterized or validated for this purpose. A more continuous cell source would also increase the throughput of this method, benefiting for example industrial screening approaches.

As emphasized before, the free drug concentration inside the cells is a more important parameter compared to the total concentration of the drug when considering the action of the drug. The distribution of drugs to pigmented tissues does not yet tell much about the effect in these tissues, as the unbound concentration of a melanin-binding drug, as

shown in this study, is much lower than the total concentration. To obtain sustained action of the drug in pigmented ocular tissues through a melanin-bound drug depot, assessing the unbound fraction is of high importance. This targeting approach has already been shown as a possibility in treating choroidal neovascularization in pigmented rats and mice [12]. The improved action was shown as elevated total drug concentrations of a high melanin binder in the pigmented uveal tract in rats. The effect of the drug was confirmed separately in pigmented mice, and the studied drug candidate was concluded to be more effective than the control drug due to higher distribution of the drug candidate into pigmented tissues caused by its higher melanin binding. Here, the total distribution of the drug to the pigmented tissues corresponded with short-term efficacy of the drug but long-term correspondence still remains unknown. Measuring unbound drug fractions in pigmented cells bridges the gap between drug distribution and drug effect in pigmented tissues, as more information can be obtained from the total concentration when understanding its relationship with the free concentration.

5. Conclusions

We demonstrated the decreased intracellular unbound fractions and increased cell uptake of melanin-binding drugs in pigmented compared to non-pigmented RPE cells. We also predicted cell uptake and unbound fractions based on melanin binding parameters from *in vitro* binding studies. The correlation between predicted and measured cell uptake demonstrated the suitability of using *in vitro* melanin binding parameters in predicting drug uptake, but other factors, such as pH partitioning inside the cells, can improve the prediction. A correlation was also found between the measured and predicted intracellular unbound fractions, but here other cellular binding seems to play an important role, and should be taken into account. All in all, our results show a wide range of values (3–4 orders of magnitude) for cellular uptake and unbound fraction in the pigmented RPE cells. The cell uptake and free concentration values indicate that melanin bound drug can form a major intracellular depot for long-term drug release within the pigmented cells. Understanding the relationship of the free and total drug concentration can help bridge the gap between drug distribution and drug action in pigmented tissues. This is important in understanding the implications of pigment binding for targeting, efficacy and toxicity of drugs.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2018.05.034.

References

[1] R.M. Ings, The melanin binding of drugs and its implications, Drug Metab. Rev. 15 (1984) 1183–1212.
[2] B.S. Larsson, Interaction between chemicals and melanin, Pigment Cell Res. 6 (1993) 127–133.
[3] B. Leblanc, S. Jezequel, T. Davies, G. Hanton, C. Taradach, Binding of drugs to eye melanin is not predictive of ocular toxicity, Regul. Toxicol. Pharmacol. 28 (1998) 124–132.
[4] A.-K. Rimpelä, M. Reinisalo, L. Hellinen, E. Grazhdankin, H. Kidron, A. Urtti, E.M. del Amo, Implications of melanin binding in ocular drug delivery, Adv. Drug Deliv. Rev. (2018) (In press).
[5] K.P. Watts, R.G. Fairchild, D.N. Slatkin, D. Greenberg, S. Packer, H.L. Atkins, S.J. Hannon, Melanin content of hamster tissues, human tissues, and various melanomas, Cancer Res. 41 (1981) 467–472.
[6] L. Pelkonen, U. Tengvall-Unadike, M. Ruponen, H. Kidron, E.M. del Amo, M. Reinisalo, A. Urtti, Melanin binding study of clinical drugs with cassette dosing and rapid equilibrium dialysis inserts, Eur. J. Pharm. Sci. 109 (2017) 162–168.
[7] L. Salminen, A. Urtti, Disposition of ophthalmic timolol in treated and untreated rabbit eyes. A multiple and single dose study, Exp. Eye Res. 38 (1984) 203–206.
[8] L. Salminen, A. Urtti, L. Periviita, Effect of ocular pigmentation on pilocarpine pharmacology in the rabbit eye. I. Drug distribution and metabolism, Int. J. Pharm. 18 (1984) 17–24.
[9] A. Urtti, L. Salminen, H. Kujari, V. Jäntti, Effect of ocular pigmentation on pilocarpine pharmacology in the rabbit eye. II. Drug response, Int. J. Pharm. Sci. 19 (1984) 53–61.
[10] N.G. Lindquist, Chapter II: melanin affinity of chloroquine and chlorpromazine examined by whole body autoradiography, Acta Radiol. 14 (1973) 19–43.
[11] A. Rosenthal, H. Kolb, D. Bergsma, D. Huxsoll, J. Hopkins, Chloroquine retinopathy in the rhesus monkey, Invest. Ophthalmol. Vis. Sci. 17 (1978) 1158–1175.
[12] S.J. Robbie, P.L. Von Leithner, M. Ju, C.A. Lange, A.G. King, P. Adamson, D. Lee, C. Sychterz, P. Coffey, Y.S. Ng, J.W. Bainbridge, D.T. Shima, Assessing a novel depot delivery strategy for noninvasive administration of VEGF/PDGF RTK inhibitors for ocular neovascular disease, Invest. Ophthalmol. Vis. Sci. 54 (2013) 1490–1500.
[13] A. Mateus, P. Matsson, P. Artursson, Rapid measurement of intracellular unbound drug concentrations, Mol. Pharm. 10 (2013) 2467–2478.
[14] A. Mateus, P. Matsson, P. Artursson, A high-throughput cell-based method to predict the unbound drug fraction in the brain, J. Med. Chem. 57 (2014) 3005–3010.
[15] A. Mateus, L.J. Gordon, G.J. Wayne, H. Almqvist, H. Axelsson, B. Seashore-Ludlow, A. Treyer, P. Matsson, T. Lundbäck, A. West, M.M. Hann, P. Artursson, Prediction of intracellular exposure bridges the gap between target- and cell-based drug discovery, Proc. Natl. Acad. Sci. 114 (2017) E6231–E6239.
[16] D.A. Smith, L. Di, E.H. Kerns, The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery, Nat. Rev. Drug Discov. 9 (2010) 929–939.
[17] A.-K. Rimpelä, M. Schmitt, S. Latonen, M. Hagström, M. Antopolsky, J.A. Manzanares, H. Kidron, A. Urtti, Drug distribution to retinal pigment epithelium: studies on melanin binding, cellular kinetics, and single photon emission computed tomography/computed tomography imaging, Mol. Pharm. 13 (2016) 2977–2986.
[18] A. Mateus, A. Treyer, C. Wegler, M. Karlgren, P. Matsson, P. Artursson, Intracellular drug bioavailability: a new predictor of system dependent drug disposition, Sci. Rep. 7 (2017) 43047.
[19] J.A. Manzanares, A.-K. Rimpelä, A. Urtti, Interpretation of ocular melanin drug binding assays. Alternatives to the model of multiple classes of independent sites, Mol. Pharm. 13 (2016) 1251–1257.
[20] L. Feeney-Burns, E.S. Hilderbrand, S. Eldridge, Aging human RPE: morphometric analysis of macular, equatorial, and peripheral cells, Invest. Ophthalmol. Vis. Sci. 25 (1984) 195–200.
[21] EMBL-EBI, ChEMBL, https://www.ebi.ac.uk/chembl/, (2018) , Accessed date: 8 February 2018.
[22] R. Oerlemans, J. van der Heijden, J. Vink, B.A.C. Dijkmans, G.J.L. Kaspers, W.F. Lems, G.L. Scheffer, I. Ifergan, R.J. Scheper, J. Cloos, Y.G. Assaraf, G. Jansen, Acquired resistance to chloroquine in human CEM T cells is mediated by multidrug resistance–associated protein 1 and provokes high levels of cross-resistance to glucocorticoids, Arthritis Rheum. 54 (2006) 557–568.
[23] M. Vezmar, E. Georges, Direct binding of chloroquine to the multidrug resistance protein (MRP): possible role for MRP in chloroquine drug transport and resistance in tumor cells, Biochem. Pharmacol. 56 (1998) 733–742.
[24] E. Mannermaa, K.-S. Vellonen, T. Ryhänen, K. Kokkonen, V.-P. Ranta, K. Kaarniranta, A. Urtti, Efflux protein expression in human retinal pigment epithelium cell lines, Pharm. Res. 26 (2009) 1785–1791.
[25] S. Nagar, K. Korzekwa, Commentary: nonspecific protein binding versus membrane partitioning: it is not just semantics, Drug Metab. Dispos. 40 (2012) 1649–1652.
[26] M. Tsuchiya, S. Hayasaka, K. Mizuno, Affinity of ocular acid-insoluble melanin for drugs in vitro, Invest. Ophthalmol. Vis. Sci. 28 (1987) 822–825.
[27] P.K. Basu, I.A. Menon, S.D. Persad, J.D. Wiltshire, Binding of chlorpromazine to cultured retinal pigment epithelial cells loaded with melanin, Lens Eye Toxic. Res. 6 (1989) 229–240.
[28] L. Pelkonen, K. Sato, M. Reinisalo, H. Kidron, M. Tachikawa, M. Watanabe, Y. Uchida, A. Urtti, T. Terasaki, LC-MS/MS based quantitation of ABC and SLC transporter proteins in plasma membranes of cultured primary human retinal pigment epithelium cells and immortalized ARPE19 cell line, Mol. Pharm. 14 (2017) 605–613.
[29] A. Sorkio, H. Hongisto, K. Kaarniranta, H. Uusitalo, K. Juuti-Uusitalo, H. Skottman, Structure and barrier properties of human embryonic stem cell-derived retinal pigment epithelial cells are affected by extracellular matrix protein coating, Tissue Eng. Part A 20 (2014) 622–634.