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Melanosomes and melanin in drug binding

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Melaniini on tumma pigmenttiaine, jota tuotetaan happamissa, lipidikalvon ympäröimissä soluelimissä, melanosomeissa. Sen tiedetään sitovan monia lääkeaineita itseensä ja näin vaikuttavan lääkeaineiden farmakokinetiikkaan. Tämän pro gradu tutkielman tarkoituksena oli tutkia kuuden lääkeaineen sitoutumista melaniiniin. Propranololin ja metformiinin sitoutumista tutkittiin sekä synteettisellä että seepian melaniinilla, kuin myös melanosomeilla pigmentoiduilla ARPE-19 -soluilla. Gansikloviirin, vorikonatsolin, kinidiinin ja siprofloksasiinin sitoutumista tutkittiin seepian melaniinilla. Sipsin isotermiä käytettiin arvioimaan B_{max} - ja K_d -arvot näille lääkeaineille.

Synteettinen ja seepian melaniini käyttäytyivät hyvin eri tavoin kokeissa. Synteettisen melaniinin huomattiin sitovan enemmän propranololia ja metformiinia kuin seepian melaniinin. ARPE-19 -solujen pigmentaatio lisäsi näiden lääkkeiden kertymistä soluihin. Propranololi, kinidiini ja siprofloksasiini sitoutuivat hyvin melaniiniin, mutta metformiinin, gansikloviirin ja vorikonatsolin sitoutuminen oli vähäistä. Sipsin isotermin huomattiin ennustavan tarkemmin voimakkaiden kuin heikkojen melaniiniin sitoutujien käyttäytymistä.

Johtopäätöksenä voidaan todeta, että valittu melaniini ja tutkittavan lääkkeen ominaisuudet vaikuttavat sitoutumiskokeiden lopputulokseen. Sipsin isotermi sopii hyvin sitoutumisen arviointiin, jos tutkittava lääkeaine sitoutuu hyvin melaniiniin.

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Melanin is a dark pigment produced in acidic, membrane-bound organelles called melanosomes. It is known to bind several drugs and have an effect on their pharmacokinetics. The aim of this thesis was to study melanin binding of six different clinical drugs. Melanin binding of propranolol and metformin was studied with synthetic and sepia melanin. ARPE-19 cells were re-pigmented with isolated porcine melanosomes, and accumulation of these two drugs was experimented. Sip's isotherm was used to estimate B_{max} and K_d values for ganciclovir, voriconazole, quinidine and ciprofloxacinin binding studies done with sepia melanin.

Synthetic melanin and sepia melanin behaved in very different ways during the studies. Synthetic melanin was found to bind drugs in higher extent than sepia melanin due to higher melanin content. Pigmentation of ARPE-19 cells increased cellular drug accumulation compared to non-pigmented cells. Propranolol, quinidine and ciprofloxacin were bound to melanin in high extents, whereas melanin binding of metformin, ganciclovir and ciprofloxacin was low. Sip's isotherm worked better with high melanin binders, than with low melanin binders.

In conclusion, melanin type and physicochemical properties of drug have major impact on melanin binding. Sip's isotherm is useful for analysing melanin binding of high melanin binders.

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Abbreviations

α-MSH	Alpha-melanocyte stimulating hormone
ARPE-19	Retinal pigment epithelial cell
ATP7A	Copper-transporting ATPase 1
CPM	Counts per minute
Dct	Dopachrome tautomerase
DHI	Dihydroxyindole
DHICA	Dihydroxyindole carboxylic acid
DMEM	Dulbecco's modified eagle medium
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GPR143	G-protein coupled receptor 143
IPE	Iris pigment epithelium
KO	Knock-out
Lamp	Lysosome-associated membrane protein
LC-MS/MS	Liquid chromatography mass spectrometry
MATP	Membrane-associated transporter protein
Melan-A	Melanoma antigen recognized by T-cells
MCR1	Melanocortin 1 receptor
MFSD12	Major facilitator superfamily domain containing 12
MRP4	Multidrug resistance-associated protein 4
NCKX5	Sodium/potassium/calcium exchanger 5
OA	Ocular albinism
OCA	Oculocutaneous albinism
PBS	Phosphate buffered saline
PCE	Pigment ciliary epithelium
Rab	Ras-related protein
rpm	Rotations per minute
Pmel	Premelanosomal protein

ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
SLC	Solute carrier
TPC2	Two pore channel 2
Tyr	Tyrosinase
Tyrp1	Tyrosinase related protein 1
V-ATPase	Vacuolar H^+ -ATPase
ZnT2	Proton-coupled zinc antiporter

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Review of the literature

1 Introduction

Melanin is a dark, polyanionic and polymeric pigment found in all forms of life in nature (Karlsson and Lindquist 2016, Cao et al. 2021). It is well known for its ability to absorb electromagnetic radiation in a wide range of frequencies (Karlsson and Lindquist 2016, Casadevall et al. 2017). In animals, melanin is found especially in skin, eye and hair, where it provides pigmentation and photoprotection against tissue damage induced by ultraviolet radiation (Ito and Wakamatsu 2008, Wasmeier et al. 2008). Additionally, melanin can act as an antioxidant and maintains cellular homeostasis by binding metal ions, free radicals, reactive species and even xenobiotics (Borovanský and Riley 2011). Many studies have demonstrated drug accumulation and retention in pigmented tissues, especially in melanin-rich ocular tissues (Leblanc et al. 1998, Rimpelä et al. 2018 a). Drug binding to melanin is an interesting phenomenon in the sense of toxicity, pharmacology and drug targeting (Leblanc et al. 1998, Rimpelä et al. 2018 a, Allen et al. 2022)

Melanin is produced and stored in melanosomes (D'Alba and Shawkey 2019). These membrane-bound organelles are found in pigmented tissues and located in melanocytes and pigment epithelial cells (Delevoye et al. 2011). Melanosomes are related to lysosomes and share some common features and disorders with them (Marks and Seabra 2001). However, melanosomes are appreciated as unique organelles with specialized properties and functions (Delevoye et al. 2011).

As melanin-synthetizing and containing organelles, melanosomes play an important role in drug distribution and retention in pigmented tissues (Rimpelä et al. 2018 a). This thesis discusses melanosomes and their effects on pharmacokinetics.

2 Melanin

2.1 Melanin types

Five different types of melanin are known to exist (Cao et al. 2021). Eumelanin and pheomelanin are found in multiple animal tissues. Both melanin types are synthetized from tyrosinase in melanosomes, but they have distinct chemical and physiological properties (García-Borrón and Olivares Sánchez 2011, Rimpelä et al. 2018a). Eumelanin is the most widely studied of melanin types (Cao et al. 2021). It is colored brown/black acts as photoprotective antioxidant (Ito and Wakamatsu 2008, Karlsson and Lindquist 2016). Pheomelanin is yellow/red, and it is known to act as pro-oxidizing photosensitizer. As eumelanin and pheomelanin are responsible for animal, including human pigmentation, this thesis focuses on these types of melanin.

Neuromelanin is produced in the brain and is closely related to eumelanin and pheomelanin (Karlsson and Lindquist 2016). It is believed to be produced from dopamine and cysteine, though this process is not well understood yet. Allomelanin and pyomelanin are less studied melanins (D'Alba and Shawkey 2019). They are found in plants and microorganisms.

2.2 Melanin synthesis

Melanin synthesis is a highly regulated, multi-step process taking place in melanosomes (Ito and Wakamatsu 2008, García-Borrón and Olivares Sánchez 2011, Rimpelä et al. 2018a). The process is also called mixed melanogenesis: it results in the mixture of eumelanin and pheomelanin (Ito and Wakamatsu 2008). Proportions of eumelanin and pheomelanin vary, but usually the proportion of pheomelanin is less than 25 % (Borovanský and Riley 2011, D'Alba and Shawkey 2019). Melanocortin 1 receptor (MC1R) signaling pathway controls expression of proteins required for eumelanin synthesis (Nasti and Timares 2015). Keratinocytes secrete alpha-melanocyte-stimulating hormone (α-MSH) as a result of exposure to ultraviolet radiation. α-MSH activates MC1R on the membrane of melanocytes. MC1R signaling pathway is activated and expression of tyrosinase (Tyr), dopachrome tautomerase (Dct) and tyrosinase-related

protein 1 (Tyrp1) is enhanced. Mutations in MC1R suppress eumelanogenesis leading to red hair phenotype producing only pheomelanin. As pheomelanin is phototoxic, this phenotype is particularly susceptible to skin damage and skin cancer.

Melanin synthesis begins with Tyr converting tyrosine to L-dopa and further to dopaquinone (figure 1) (Ito and Wakamatsu 2008, Rimpelä et al. 2018a). Tyrosinase is an essential enzyme for melanogenesis (Le et al. 2021). Defects in its function lead to severe form of oculocutaneous albinism (OCA). OCA is a group of inheritable pigmentation disorders causing hypopigmentation in skin, hair and eyes (Grønskov et al. 2007).

Next steps of melanin synthesis happen in three distinct phases (figure 1) (Ito and Wakamatsu 2008, Rimpelä et al. 2018a). At phase 1 dopaquinone forms cysteinyldopa with cysteine, which is required to be present at least at 0.13 µM concentration. Cysteine is transported to melanosomes by major facilitator superfamily domain containing 12 (MFSD12) (Adelmann et al. 2020). Next, at phase 2, cysteinyldopa is oxidized by dopaquinone and pheomelanin is formed (Ito and Wakamatsu 2008, Rimpelä et al. 2018 a). This reaction requires concentration of cysteinyldopa to be more than 9 µM.

Phase 3, synthesis of eumelanins, begins when cysteinyldopa and cysteine are consumed (figure 1) (Ito and Wakamatsu 2008, Rimpelä et al. 2018a). First steps of the phase are spontaneous: cyclodopa is produced from dopaquinone and converted further to dopachrome. Then, dopachrome is used to form eumelanin monomers dihydroxyindole (DHI) and dihydroxyindole carboxylic acid (DHICA). Both monomers can be formed spontaneously, but usually formation of DHICA is catalyzed by Dct (Rimpelä et al. 2018a). DHICA has stronger antioxidant properties, and its proportion is found to be higher than DHI proportion in natural eumelanin. Tyrp1 is believed to catalyze DHICA polymerization to final eumelanin product, but its exact role in melanogenesis is yet to be unraveled (Le et al. 2021). As Dct and Tyrp1 support eumelanogenesis, defects in their functions cause different types of OCA.

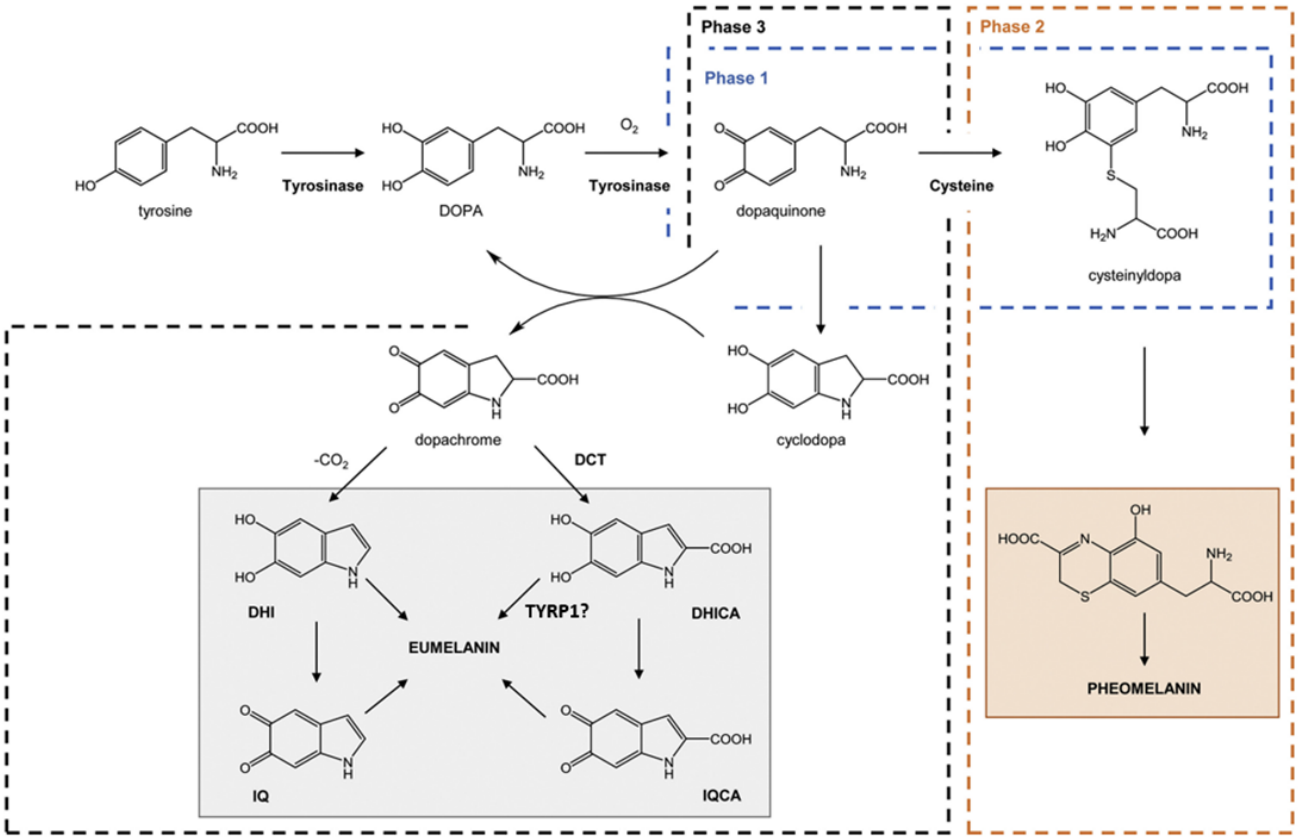

Figure 1. Mixed melanogenesis happens in three phases and produces both pheomelanin and eumelanin. Modified from (Rimpelä et al. 2018 a).

As a result of mixed melanogenesis, eumelanin and pheomelanin are believed to form granules with pheomelanin core covered with eumelanin (figure 2) (Ito and Wakamatsu 2008). This casing model is proved with neuromelanin, and more evidence is found in the studies conducted with cultured human skin and uveal melanocytes.

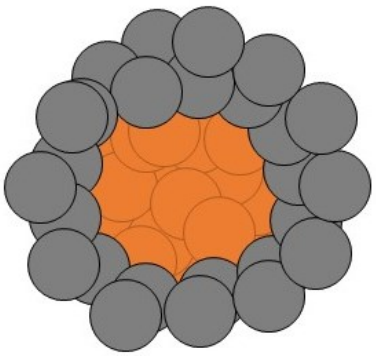

Figure 2 According to casing model pheomelanin forms the core of the particle, which is covered with eumelanin. Figure is drawn according to (Ito and Wakamatsu 2008).

2.3 Pigmented tissues

Pigment epithelial cells and melanocytes are the cells producing melanin in their melanosomes (Colombo et al. 2011). Pigment epithelial cells are found in the eye: in iris pigment epithelium (IPE), retinal pigment epithelium (RPE) and pigment ciliary epithelium (PCE) (figure 3) (Rimpelä et al. 2018a). These cells originate embryonically from neuroectoderm. Melanin production and tyrosinase activity is low or zero in adult RPE. Melanin contents in RPE is known to decrease with age (Różanowska 2011). Similar age-related degradation is not observed in IPE melanosomes

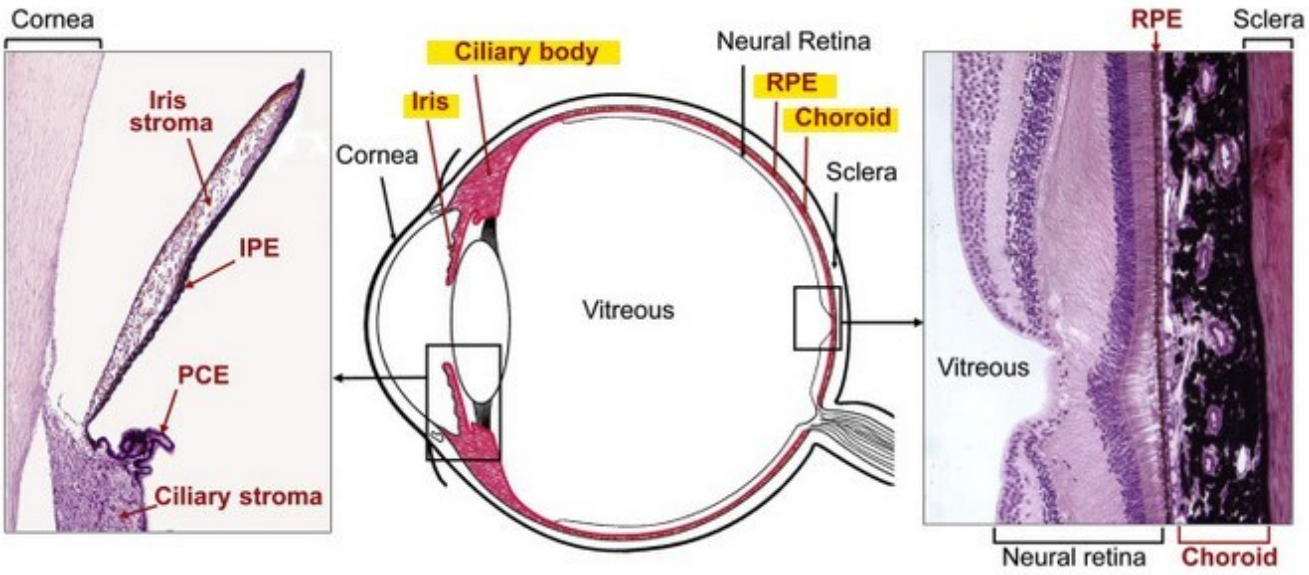

Figure 3 Structure of the eye, pigmented tissues are highlighted in red. Pigment epithelial cells form IPE, RPE and PCE. Melanocytes are found in choroid, iris stoma and ciliary body. Modified from (Rimpelä et al. 2018a).

The embryonic origin of melanocytes lies in neural crest, and the cells can be classified as classical and non-classical melanocytes based on the embryogenic pathway their development follows (Colombo et al. 2011). Classical melanocytes follow dorsolateral pathway and are found in skin tissues (epidermis, dermis and follicles). Epidermal melanocytes interact with keratinocytes and provide them with melanin. Non-classical melanocytes follow other embryogenic pathways. They are found in the eye (iris stroma, ciliary body and in the choroid, see figure 3), inner ear, brain, heart and adipose tissue (Colombo et al. 2011, Różanowska 2011, Rimpelä et al. 2018a).

Different skin, hair and eye colors are due to different amounts of eumelanin and pheomelanin. For example, in the iris the total melanin amounts vary between 0.03 µg/iris in blue eyes and 32.2 µg/iris in brown eyes (Różanowska 2011). In addition to photoprotection and pigmentation, melanin has an important role in maintaining homeostasis in different tissues. In the inner ear, melanin protects the tissues from reactive oxygen species (ROS) created by high intensity noise and regulates Ca^{2+} -balance in haircells (Colombo et al. 2011). Melanin regulates ROS and Ca^{2+} levels also in cardiac tissues (Levin et al. 2009). Imbalance in these levels has been associated with development of arrhythmias. Adipose tissues in morbidly obese patients are found to produce melanin (Page et al. 2011). Production of melanin is believed to be a response to inflammation and oxidative stress related to obesity.

2.4 Drug binding to melanin

Melanin is known to bind multiple drugs (Leblanc et al. 1998, Pelkonen et al. 2017a, Rimpelä et al. 2018a). Especially lipophilic and basic compounds are strong melanin binders (Leblanc et al. 1998). Many in vivo studies have demonstrated how drugs accumulate in higher extents and are retained longer in pigmented tissues than in albino tissues (Salazar et al. 1976, Urtti et al. 1984, Nagata et al. 1993). Melanin binding can prolong the action of the drug (Salazar et al. 1976). For example, in the experiments by Salazar et al. (1976), mydriasis caused by atropine lasted remarkably longer time in the eyes of pigmented rabbits than in the eyes of albino rabbits. Atropine was bound to melanin of pigmented eye and was released slowly. Melanin binding can also weaken the effect of the drug (Nagata et al. 1993). Timolol and pilocarpine lowered intraocular pressure in albino rabbits, but similar effect was not seen in pigmented rabbits. The drugs were bound to melanin in pigmented ocular tissues and were not able to reach their intended binding sites to lower the intraocular pressure.

Chloroquine binding is considered as an example of adverse effects of melanin binding (Leblanc et al. 1998, Schroeder and Gerber 2014). It is known to bind in high amounts to melanin, and its retention time in pigmented ocular tissues is long. Long exposure to chloroquine is known to cause oculotoxic side effects, such as retinopathy. However, it is noteworthy, that generally no

conclusions about ocular toxicity of a drug can be drawn based on melanin binding of the compound (Leblanc et al. 1998).

3 Melanosomes

Many intermediate products of melanin synthesis are oxidative and cytotoxic (Borovanský and Riley 2011, Delevoye et al. 2011). Thus, to protect the other cellular components, melanin is produced and stored in melanosomes. Healthy melanosome membrane is nearly impermeable, but in some diseases, for example in melanoma, the membrane becomes defective and leaks toxic compounds to cytosol (Chen et al. 2009, Borovanský and Riley 2011). This feature is studied to be utilized in melanoma treatment.

Melanosomes contain mostly melanin and proteins, but also other substances such as lipids, carbohydrates and inorganic compounds are found (Borovanský and Riley 2011). Melanosome shape, composition and amounts of melanin vary highly depending on the origin of melanosomes. For example, melanosomes in RPE are ellipsoid in shape and contain mainly eumelanin, but melanosomes in skin and iris stoma are spherical in shape and contain mixture of eumelanin and pheomelanin (Różanowska 2011, Rimpelä et al. 2018 a). In some contexts, melanosomes can be classified as eumelonosomes and pheomelanosomes based on melanin they mainly produce (Borovanský and Riley 2011, D'Alba and Shawkey 2019). However, this classification is controversial, as eumelanin is dominant in nature, and pure pheomelanin is observed only rarely (Borovanský and Riley 2011).

3.1 Relation to lysosomes

Melanosomes are lysosome-related organelles (Le et al. 2021). Lysosome-related organelles are membrane bound organelles, that share common features with lysosomes, and are derived from endosomes (Wasmeier et al. 2008, Le et al. 2021). Vacuolar H^+ -ATPase (V-ATPase), MFSD12 and lysosome-associated membrane proteins 1 and 2 (Lamp1, Lamp2) are examples of proteins common to both lysosomes and melanosomes (Orlow et al. 1993, Basrur et al. 2003, Adelmann et al. 2020). Intraluminal pH of melanosomes is acidic, like it is in lysosomes (Ancans et al. 2001, Derendorf 2020). Stage I melanosomes are also called early endosomes, and they diverge to both stage II melanosomes and late endosomes (Le et al. 2021). Mechanism of this phenomenon

is not known, but G-protein coupled receptor 143 (GPR143) signaling is hypothesized to have a driving role in segregation of melanosomes and endosomes. L-dopa, the first intermediate product of melanin synthesis, seems to be a ligand for this receptor (Lopez et al. 2008). GPR143 is defective in ocular albinism type 1 (OA1), which causes hypopigmentation in ocular tissues (Le et al. 2021, Neveu et al. 2022). Diseases causing dysfunction of lysosome-related organelles affect also on melanosomes (Marks and Seabra 2001). Oculocutaneous albinism is observed in Hermansky-Pudlak syndrome. Partial albinism is a symptom of Griscelli syndrome and Chediak Higashi syndrome.

3.2 Melanosome biogenesis

Melanosomes maturation happens in four stages (figure 4) (Delevoye et al. 2011). Melanosomes at stage I and II are called premelanosomes. They lack tyrosinase activity and thus melanin synthesis is absent. Stage I melanosomes contain intraluminal vesicles and irregular premelanosome protein (Pmel) fibrils. At stage II, protein matrix consistent of Pmel reaches its form and gives eumelanosome its shape. Pheomelanosomes don't develop similar matrix, and their shape is irregular (D'Alba and Shawkey 2019). Melanin synthesis begins at stage III (Delevoye et al. 2011). In eumelanosomes, melanin is stored and arranged by protein fibrils. Finally, at stage IV, melanosomes are fully matured.

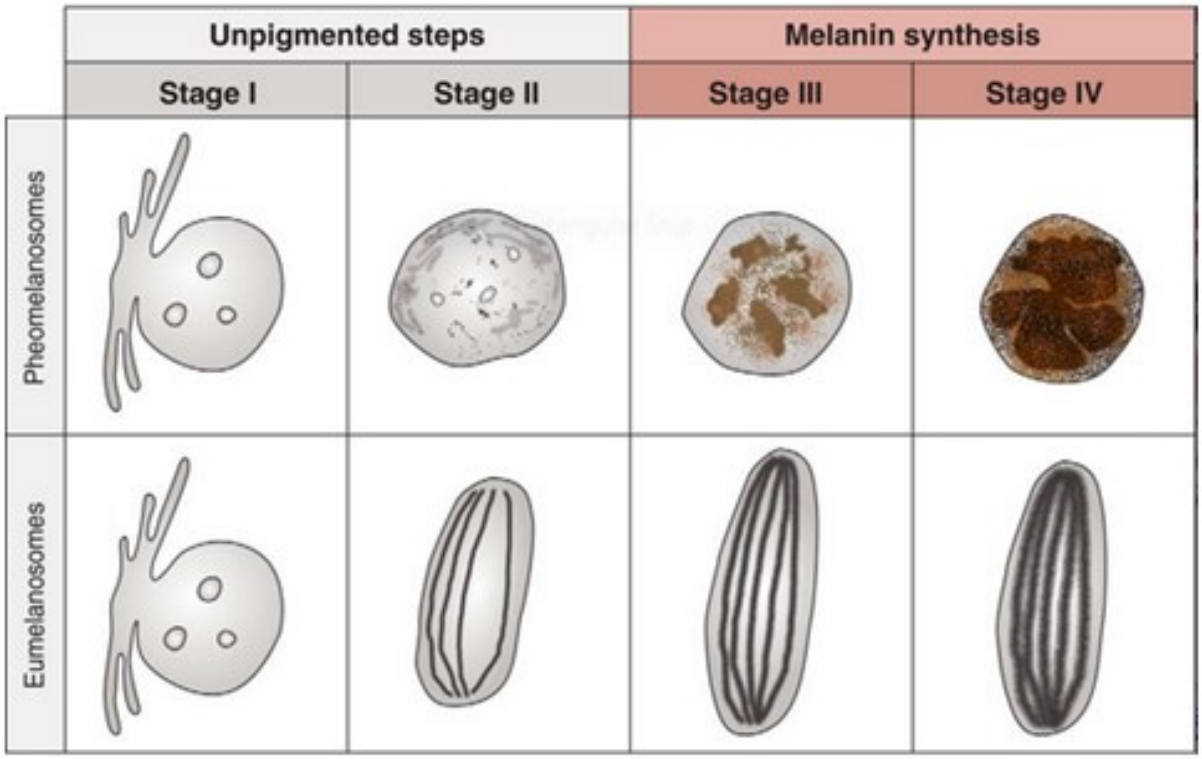

Figure 4. Melanosomes mature in four stages. Modified from D'Alba and Shawkey 2017

In RPE, mature melanosomes are transported to apical processes of the cells, near to the photoreceptors, where they provide photoprotection (Van Gele and Lambert 2011). RPE melanosomes are also believed to be involved in degradation of photoreceptor outer discs, as proteolytic enzyme cathepsin D is identified in RPE melanosomes (Azarian et al. 2006).

In epidermis, mature melanosomes are first transported to periphery of dendritic melanocytes and then transferred to keratinocytes (Wu and Hammer 2014, Moreiras et al. 2021). The exact mechanism for transfer is not known yet, but some alternative mechanisms have been hypothesized:

a) Dendrite tip of melanocyte is phagocytosed by keratinocyte.
b) Melanocyte and keratinocytes membrane fuse, and melanosomes are transferred along nanotube formed by the fusion.
c) Melanocytes shed melanosome containing vesicles, which are phagocytosed by keratinocytes.
d) Melanin is exocytosed from melanocytes and phagocytosed by keratinocytes.

3.3 Melanosomal proteins

Approximately 1500 proteins in total are incorporated in melanosome proteome (Chi et al. 2006). Some of these proteins are cell type or maturation stage specific, but 100 proteins are common to all stages of maturation of melanosomes. Basrur et al. (2003) identified 68 proteins in early-stage melanosomes in MNT1 -cells, of which six were known to be melanosome-specific, whereas 56 of these proteins were common with other organelles such as lysosomes (e.g., V-ATPase, Lamp1, Lamp2). Azarian et al. (2006) identified over 100 proteins in mature porcine RPE melanosomes. Twelve of these proteins were same than those identified by Basrur et al.

A recent discovery was made by Beyers et al. (2022), when OCA7 was found to be localized to melanosome membrane and having a role in normal Pmel processing and intraorganelle pH regulation. OCA7 -gene was knocked out (KO) in MNT1 -cells. Compared to wild type, OCA7-KO exhibited fewer melanosomes which had fully processed Pmel fibers. Fluorescence measurements revealed melanosomes in OCA7-KO -cells to be more acidic compared to melanosomes in wild type cells.

Proteins have multiple functions in melanosomes, and defects in these functions can cause different disorders (table 1). Six proteins (Tyr, Tyrp1, Dct, Pmel, GPR-143 and Mart1) are melanosome-specific (Chi et al. 2006). Several transporters regulate pH (e.g., V-ATPase, SLC45A2 and ion homeostasis (e.g., SCL24A5) in melanosomes (Wiriyasermkul et al. 2020). Rab GTPases, 21 proteins in total, are involved in melanosome functions (Fukuda 2021). They are associated in protein sorting and trafficking to melanosomes (e.g., Rab32, Rab38), transport (e.g., Rab27a) and transfer (e.g., Rab11A and Rab11B) of the organelles.

Table 1. Examples of melanosomal proteins and transporters.

Protein	**Function in melanosomes**	**Disorder**	**Reference**
Tyr	Melanin synthesis	OCA1	Ito and Wakamatsu 2008
Tyrp1	Melanin synthesis	OCA3	Ito and Wakamatsu 2008

Protein	**Function in melanosomes**	**Disorder**	**Reference**
Dct	Melanin synthesis	OCA8	Ito and Wakamatsu 2008
Pmel	Melanin organization, melanosome shape	pigmentary glaucoma	Le et al. 2021
GPR-143	Segregation from endosomes	OA1	Le et al. 2021
Melan-A (Mart1)	PmelL/GPR-143 chaperone?		Hoashi et al. 2005, Le et al. 2021
V-ATPase	pH regulation: acidification		Wiriyasermkul et al. 2020
TPC2	pH regulation: acidification		Ambrosio et al. 2016
MATP (SLC45A2)	pH regulation: neutralization	OCA4	Wiriyasermkul et al. 2020
OCA2	pH regulation: neutralization	OCA2	Wiriyasermkul et al. 2020
OCA7	pH regulation, Pmel processing	OCA7	Beyers et al. 2022
NCKX5 (SCL24A5)	Ca^{2+} and Na^{+} transporter	OCA6	Chi et al. 2006, Zhang et al. 2019
ATP7A	Cu^{2+} transporter	Menkes disease	Wiriyasermkul et al. 2020
ZnT2 (SLC30A2)	Zn^{2+} transporter		Wiriyasermkul et al. 2020
MFSD12	Cysteine uptake		Adelmann et al. 2020
MRP4	Organic anion transporter		Azarian et al. 2006
Rab11a, Rab11b, Rab27a, Rab,32, Rab38	Protein sorting and trafficking, melanosome motility	Griscelli syndrome (Rab27a)	Fukuda 2021

Protein	Function in melanosomes	Disorder	Reference
Myosin Va	Melanosome transport		Wasmeier et al. 2008
Lamp1, Lamp2			Orlow et al. 1993, Basrur et al. 2003
Cathepsin D	Proteolytic enzyme		Azarian et al. 2006

ATP7A, copper-transporting ATPase 1; Dct, dopachrome tautomerase; GPR143, G-protein coupled receptor 143; Lamp, lysosome-associated membrane protein; MATP, membrane-associated transporter protein; Melan-A, melanoma antigen recognized by T-cells; MCR1, melanocortin 1 receptor; MFSD12, Major facilitator superfamily domain containing 12; MRP4, multidrug resistance-associated protein 4; NCKX5, sodium/potassium/calcium exchanger 5; OA, ocular albinism; OCA, oculocutaneous albinism; Pmel, premelanosomal protein; Rab, ras-related protein; SLC, solute carrier; TPC2, two pore channel 2; Tyr, tyrosinase; Tyrp1, tyrosinase related protein 1; V-ATPase, Vacuolar H^+ -ATPase; ZnT2, proton-coupled zinc antiporter

3.3.1 Transporters

Transporters localized on the membrane of the melanosomes play an important role in melanogenesis and pigmentation (Wiriyasermkul et al. 2020). Especially pH regulation mediated by membrane associated transporters is critical: Pmel17 fibrils are formed in acidic conditions, but melanin synthesis requires neutral environment (Ancans et al. 2001, Dean and Lee 2019).

V-ATPase and two pore channel protein 2 (TPC2) acidify melanosomes (Ambrosio et al. 2016, Wiriyasermkul et al. 2020). V-ATPase is an H+ influx pump (Wiriyasermkul et al. 2020). It is found in melanosomes at all stages of maturation, and in many other organelles, such as lysosomes. TPC2 is expressed at stages III and IV, and its action is believed to be due to Ca^{2+} extrusion (Ambrosio et al. 2016).

pH neutralization is due to activity of membrane associated transporter protein (MATP, also known as solute carrier family 45 member 2 (SLC45A2)) and OCA2 proteins, both expressed in melanosomes at stages III and IV (Le et al. 2020, Wiriyasermkul et al. 2020). MATP acts as an H^+

and sucrose efflux transporter, OCA2 extrudes Cl^- ions. Mutations in MATP and OCA2 cause oculocutaneous albinism types IV and II, respectively.

Sodium/potassium/calcium exchanger 5 (NCKX5, or SLC24A5) maintains Ca^{2+} levels favorable for normal Pmel fibrillation in melanosomes, but its subcellular localization is unclear (Chi et al. 2006, Zhang et al. 2019). Chi et al. (2006) have localized the transporter to melanosomes. Zhang et al. (2019) localized it rather to mitochondria and trans-Golgi network, from where Ca^{2+} is transferred to melanosomes. However, loss of NCKX5 reduces Ca^{2+} levels in melanosomes and thus compromises normal formation of Pmel fibrils and pigmentation, causing OCA6 (Zhang et al. 2019).

Cu^{2+} and Zn^{2+} are essential ions for normal functions of tyrosinase (Cu^{2+}) and Dct (Zn^{2+}) (Wiriyasermkul et al. 2020). Cu^{2+} is transported to melanosomes by ATP7A. Malfunctions in the protein leads to lethal multisystemic disorder Menkes disease (Tümer and Møller 2010). The transport mechanism of Zn^{2+} remains still unclear, but ZnT2 (SLC30A2) has been hypothesized to supply melanosomes with zinc (Wiriyasermkul et al. 2020). Cysteine, needed for pheomelanin synthesis, is transported to melanosomes and lysosomes by MFSD12 (Adelmann et al. 2020). Suppression of the transporter seems to cause darker pigmentation in humans and mice.

Multidrug resistance-associated protein 4 (MRP4), was the only drug transportation related transporter found from melanosomes by Azarian et al. (2006). MRP4 is a transporter for multiple drugs, such as hydrochlorothiazide, adefovir (antiviral), methotrexate, and sildenafil (Berthier et al. 2019).

3.4 Melanosome pH

As melanosomes are related to lysosomes, their intraorganellar pH can be expected to be acidic (Ancans et al. 2001). Low pH has been determined from melanosomes from B16 murine melanoma (pH 3.0–4.6) and skin melanocytes from mice and human (Bhatnagar et al. 1993, Puri et al. 2000, Ancans et al. 2001). The pH measurements of the studies were based on fluorescent

dyes, which can be problematic, since the dark color of the melanin may affect the fluorescence intensity (Rimpelä et al. 2018a).

Bhatnagar et al. (1993) and Puri et al. (2000) suggested that melanin synthesis requires acidic environment. Ancans et al. (2001) demonstrated different results: tyrosinase activity increased when melanosomes were neutralized using proton pump inhibitors. The group concluded that pH 6.8 is ideal for tyrosinase activity. The group also noticed that increase in pH also increases eumelanin/pheomelanin ratio. Wakamatsu et al. (2017) have also reported similar effects of pH to the ratio of eumelanin and pheomelanin.

Fuller et al. (2001) have concluded melanosomes from light skinned Caucasians being acidic, while melanosomes from dark skin with African origins are more neutral and more favorable to pigmentation. Melanocytes were treated with ammonium chloride, ionophores (H^+ is changed to Na or K) or proton pump inhibitor bafilomycin to neutralize pH. Treatments increased tyrosinase activity in Caucasian melanocytes, but similar increase was not observed in African melanocytes. The group confirmed the acidity of Caucasian melanosomes by staining the melanosomes with fluorescent dye acridine orange. Acridine orange is weakly basic and accumulates to acidic organelle (Fuller et al. 2001). It emits orange color at high concentrations, and green color at low concentrations. Caucasian melanocytes were colored orange, but African melanocytes were slightly green.

Luminal pH of melanosomes changes during the maturation process (Ancans et al. 2001, Wiriyasermkul et al. 2020). Premelanosomes at stages I and II (figure 4) are acidic, contributing to normal formation of Pmel fibrils (Dean and Lee 2019). Proton influxing V-ATPase is expressed already in stage I melanosomes (Wiriyasermkul et al. 2020). Neutral pH is a requirement for tyrosinase activity, and melanosomes need to be neutralized (Ancans et al. 2001, Wiriyasermkul et al. 2020). Neutralizing transporters MATP and OCA2 are expressed in stages III and IV, accompanied by acidifying V-ATPase and TPC2 (Wiriyasermkul et al. 2020). Melanin synthesis and melanosome maturation takes place at these stages (D'Alba and Shawkey 2019). pH in RPE melanosomes has not been determined yet, but as mature RPE melanosomes don't show

tyrosinase activity and melanogenesis, they are expected to be acidic (Różanowska 2011, Rimpelä et al. 2018 a).

3.4.1 Ion trapping

Ion trapping is a phenomenon observed in acidic membrane-bound organelles such as lysosomes and melanosomes (Rimpelä et al. 2018 a, Derendorf 2020). Basic compound is in unionized form in cytosol with pH approximately 7.4 (figure 5) (Derendorf 2020). If compound possesses adequate lipophilicity, can pass passively across the lipid membrane in unionized form. When it enters acidic lumen (pH approximately 5.0 in lysosomes) of the organelle, it gets ionized. Ionized compound can't easily pass the membrane back to cytosol and gets "trapped" into the acidic environment.

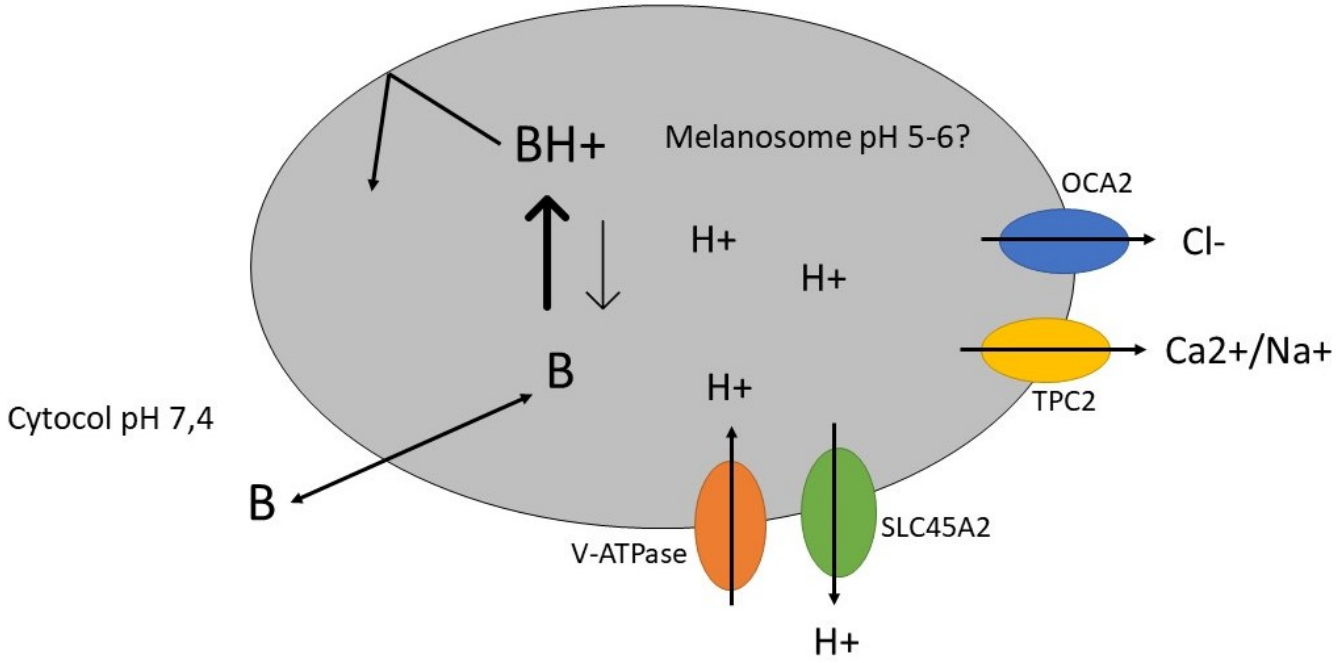

Figure 5. Basic compound (B) enters the melanosome and ionizes in acidic environment. Ionized form (BH+) gets "trapped" inside of the organelle. V-ATPase (orange), NCKX5 (SCL24A5) (green), TPC2 (yellow) and OCA2 (blue) regulate pH of melanosome.

Derendorf (2020) estimated computationally the accumulation of hydroxychloroquine and azithromycin to lysosomes. Hydroxychloroquine was estimated to accumulate in acidic lysosomes in 56 000 times higher extent than in neutral cytosol. Lysosomal accumulation of azithromycin was calculated to be 52 000 times higher than accumulation in cytosol.

Chloroquine is also known to accumulate to lysosomes as a result of ion trapping (Rimpelä et al. 2018a). It is also known to be a strong melanin binder (Schroeder and Gerber 2014). Rimpelä et al. (2018a) simulated chloroquine concentrations in RPE-choroid after intravitreal injection. They also included the effect of ion trapping to the simulation by setting outward permeability of melanosomes low. Ion trapping had increasing effect on free and total chloroquine concentrations in RPE-choroid.

4 The effect of melanosomal properties on drug distribution

As melanosomes produce and store melanin, they have an important role in drug distribution and retention in pigmented tissues (Rimpelä et al. 2018 a). Melanosomes contain both eumelanin and pheomelanin (Borovanský and Riley 2011). Especially RPE melanosomes contain mostly eumelanin, which ability to bind drugs is well studied (Różanowska 2011, Rimpelä et al. 2018 a). Pheomelanin, in turn, seems to have low affinity to drugs, and its proportion in melanosomes is low, usually less than 25 % (Mårs and Larsson 1999, Borges et al. 2003, Borovanský and Riley 2011). Thus, drug accumulation in pigmented tissues, especially in ocular tissues, is expected to be largely due to drug binding to eumelanin (Pitkänen et al. 2007).

Melanin binding is not the only way melanosomes affect drug distribution (Rimpelä et al. 2018 a, Bahrpeyma et al. 2022). Bahrpeyma et al. (2022) studied retention of few drugs in melanin, melanosomes, and re-pigmented cells. The group found that the drugs were released faster from pure melanin than from melanosomes: melanosome membrane restricts drug dissociation from the organelle Ion trapping takes place in acidic melanosomes enhancing higher drug accumulation and longer retention time in pigmented tissues (Rimpelä et al. 2018 a, Derendorf 2020).

In addition to melanin binding and ion trapping, melanosomes express multiple transporters, including MRP4 (Azarian et al. 2006). MRP4 is associated in pharmacokinetics of multiple drugs (Berthier et al. 2019).

Experimental part

5 Aims of the studies

The aims of these studies are to inspect melanin binding on six different drugs, including ganciclovir, voriconazole, quinidine and ciprofloxacin studied in a wide range of concentrations. Maximum binding capacity and dissociation constants are estimated using Sip's isotherm. Melanin binding of propranolol and metformin is studied in single concentration with synthetic melanin and melanin from *Sepia officinalis* (sepia melanin), and in re-pigmented retinal pigment epithelial cells (ARPE-19).

6 Materials and methods

6.1 Materials

Six compounds were chosen for binding studies: ganciclovir, voriconazole, quinidine, ciprofloxacin and radiolabeled propranolol and metformin (3H and 14C labels, respectively). Table 2 introduces these compounds and how they were used in the experiments.

Table 2. The information of drugs used in the studies.

Compound	**Provider information**	**Radiolabel**	**Experiment/ analytical methods**	**Exposure concentration**
Propranolol	P0884, Sigma-Aldrich (non-labeled), Net515250UC, PerkinElmer (labeled)	no label and H3	Melanin binding, melanosome binding/ scintillation counter	1 µM 5 µM
Metformin	MC-2043, Hartmann Analytic	C14	Melanin binding, melanosome binding/ scintillation counter	1 µM 5 µM
Ganciclovir	G2536, Sigma-Aldrich	-	Melanin binding/LC-MS/MS	0.5 – 500 µM
Voriconazole	32,483, Fluka	-	Melanin binding/LC-MS/MS	0.5 – 500 µM

Compound	Provider information	Radiolabel	Experiment/ analytical methods	Exposure concentration
Quinidine	22600, Sigma-Aldrich	-	Melanin binding/LC-MS/MS	0.5 – 500 µM
Ciprofloxacin	17850, Fluka	-	Melanin binding/LC-MS/MS	0.5 – 500 µM

6.2 Synthetic melanin testing

Synthetic melanin (M8631, Merck, Sigma-Aldrich) was suspended to concentration 2 mg/ml with phosphate buffered saline (1xPBS, Gibco). To observe the behavior of synhtetic melanin, series of samples (400 µl) was collected and treated according to table 3.

As a first step, the samples were either sonicated (samples 1, 2 and 5) or incubated at room temperature without any sonication (samples 3 and 4). Next, the melanin was spun down with centrifuge (14 000 rpm, 20 minutes, +4 °C, all samples) and the colors of the supernatants were inspected. After this inspection, the supernatants were removed and the remaining melanin pellets were resuspended with 1XPBS. The resuspended samples were either sonicated (samples 1 and 4) or incubated at room temperature (samples 2, 3 and 5). Finally, all samples were taken to the centrifuge, melanin was spun down and the colors of the supernatants were inspected, again.

Table 3. Treating of synthetic melanin samples.

Sample	1st step	2nd step	3rd step	4th step
1	Waterbath sonicator, 37 °C, 15 min	centrifuge + supernatant changed to the fresh 1xPBS	Waterbath sonicator, 37 °C, 15 min	centrifuge
2	Waterbath sonicator, 37 °C, 15 min	centrifuge + supernatant changed to the fresh 1xPBS	incubation at room temperature	centrifuge
3	incubation at room temperature	centrifuge + supernatant changed to the fresh 1xPBS	incubation at room temperature	centrifuge
4	incubation at room temperature	centrifuge + supernatant changed to the fresh 1xPBS	Waterbath sonicator, 37 °C, 15 min	centrifuge
5	probe sonicator, 4 x 15 seconds, 38 % amplitudes	centrifuge + supernatant changed to the fresh 1xPBS	incubation at room temperature	centrifuge

6.3 Melanin binding experiments with propranolol and metformin

Propranolol and metformin solutions (2 µM) were used in melanin binding studies. Cold propranolol solution (4 µM) was prepared in 1XPBS, and H3-labeled propranolol (43 µM, 20.6 Ci/mmol) was diluted to 43 nM with 1xPBS. 2 µM propranolol test solution was prepared by

mixing cold propranolol (4 µM), H3-labeled propranolol (43 nM) and 1XPBS in ratio 2:1:1. C14-labeled (0.8 mM, 116 mCi/mmol) metformin solution was diluted with 1xPBS to concentration of 2 µM.

Melanin, either synthetic or sepia melanin (M2649, Merkc, Sigma-Aldrich), was suspended at 2 mg/ml concentration with 1xPBS and possible aggregates were dispersed with probe sonicator (SONICS Vibra cell) by using four 15 seconds cycles (38 % amplitudes). The excess suspension was stored at -20 °C for later use. First, two binding experiments were conducted using synthetic melanin, and, after testing synthetic melanin as described earlier, the next three experiments were performed by using sepia melanin.

Equal volumes (75 µl) of test compound solution and melanin suspension were combined to Eppendorf tubes in three replicates. The mixtures were incubated at 37 °C by mixing at 250 rpm for 20 hours. After the incubation, the melanin was separated from supernatant by centrifuge (Mikro 22 R, Hettich) at 14 000 rpm, 20 minutes, +4 °C. The supernatant was collected carefully, and the melanin pellet was dissolved with 500 µl of Solvable™ (6NE9100, Perkin Elmer) by mixing it at 55 °C for an hour. Samples were collected from both supernatant and solvated melanin pellet to 24-well plates, and Ultima Gold scintillating fluid (Perkin Elmer) was added in ratio 1:4 (sample:scintillating fluid). The control samples were taken from the exposure solutions and mixed with scintillating fluid in the same manner. The plates were sealed tightly and were analyzed next day with MicroBeta 2 - 2450 Microplate counter (Perkin Elmer).

The bound fraction of drug was calculated by equation 1 as a ratio of measured counts per minute (CPM) in the melanin sample and CPM in the whole sample (melanin + supernatant).

$$melanin\ binding\ \% = \frac{CPM_{melanin}}{CPM_{melanin} + CPM_{supernatant}} * 100\ \% \quad (1)$$

6.4 ARPE-19 cells and re-pigmenting with porcine melanosomes

Retinal pigment epithelial cells (ARPE-19) (ATCC, USA) were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco BRL 31330-038) supplemented with 10 % fetal bovine serum (FBS, 10270-106, Gibco) 2 mM L-glutamine (BE17-605E, BioWhittaker, Lonza), 100 U/ml penicillin and 100 µg/ml streptomycin (DE17-602E, BioWhittaker, Lonza). The growing conditions were 37 °C and 5 % CO_2.

The cells were inspected and passaged weekly. Medium was removed and the cells were washed gently with 1xPBS. The cells were detached by incubating them with trypsin-EDTA (Gibco, 15400-054) for five minutes at 37 °C and 5 % CO_2. After the incubation trypsin was inactivated with growth medium and the cells were spun down with centrifuge (3 minutes, 1200 rpm). Medium was removed from the top of the cells, and they were resuspended with some fresh medium. Appropriate volumes of cell suspension were measured to the new cell growth bottles and more medium was added. The cells were split commonly in ratio 1:8, when passaged for maintenance.

Cell pigmenting was performed according to Hellinen et al. (2019). The cells were seeded to 96-well plate (20 000 cells/well, which equals 62 500 cells/cm2) on a day before pigmentation. Porcine melanosomes, isolated as described by Pelkonen et al. (2016), were suspended with 1xPBS and melanin concentration was measured spectrophotometrically at wavelength 595 nm (Wallac Victor^2 1420 Multilabel counter, Perkin Elmer). The standards (0,00, 0,05, 0,10, 0,25, 0,50 and 1,00 mg melanin/ml) were prepared by diluting 2 mg/ml synthetic melanin suspension with 1xPBS. Additionally, one standard curve at equal concentrations was prepared from sepia melanin. However, this curve was only compared with standard curve prepared from synthetic melanin, and it was not used to quantify melanosomes.

The melanosome suspension was added to the wells, so that either 0, 7, 20 or 68 µg melanin per well was dosed. According to Hellinen et al. (2019) the dose of 68 µg melanin/well results to ARPE 19-mel cells with melanin contents equivalent to porcine RPE-cells. Thus, this dose (68 µg/well) can be considered to respond 100 % pigmentation rate, 20 µg/well responds 30 % and 7 µg/well 10 % pigmentation rate.

The cells and melanosomes were co-incubated for a week at cell growth conditions. During this time, the medium was mixed gently with pipette few times, and it was changed once to remove melanosomes not taken up by the cells.

6.5 Cell uptake experiments with propranolol and metformin

The medium was removed from the top of the pigmented cells, and cells were washed with 1xPBS to remove the rest of the melanosomes not taken up by the cells. Exposure solution (100 µl) containing either radiolabeled propranolol or metformin prepared in cell growth medium (without antibiotics) at 5 µM concentration, were added to the cells. The cells were incubated for 20 hours, at +37 °C and 5% CO_2.

After incubation, supernatant was carefully collected from the top of the cells. The cells were washed with 100 µl of 1xPBS. This washing PBS was discarded, and fresh 1xPBS was added to the wells for an upcoming spectrophotometric assay. The melanin amounts in the pigmented cells were quantified spectrophotometrically at wavelength 595 nm with Wallac Victor^2 1420 Multilabel counter.

After spectrophotometric assay, the 1xPBS was removed, and the cells were dissolved with Solvable™ (100 µl per well) by incubating at 55 °C for an hour. Samples from the supernatant and dissolved cells were collected to 24-well plates, and scintillating fluid was added in ratio 1:4 (sample:scintillating fluid). The plates were sealed and analyzed with MicroBeta 2 - 2450 Microplate counter.

The cellular drug uptake was calculated by equation 2 as a ratio of CPM in the cell pellet and CPM in the whole sample (pellet + medium).

$$Cellular\ uptake\ (\%) = \frac{CPM_{cells}}{CPM_{cells}+CPM_{medium}} * 100\% \quad (2)$$

The melanosomal uptake was calculated by equation 3. Drug binding to non-pigmented ARPE-19 (0 %) cells is subtracted as a background from drug binding to pigmented ARPE-19mel (10 % and 30 %) cells.

$$Melanosomal\ drug\ uptake\ (\%) = Cellular\ uptake(\%)_{ARPE-19mel} - Cellular\ uptake\ (\%)_{ARPE-19} \quad (3)$$

6.6 Melanin binding experiments with ganciclovir, voriconazole, quinidine and ciprofloxacin

10 mM stock solutions were prepared from each drug: ganciclovir, voriconazole and quinidine were dissolved at dimethyl sulfoxide (DMSO, D8418, Sigma-Aldrich) and ciprofloxacin at 0.1 M HCl (milli-Q water and 37 % HCL, 20252.335, VWR). Intermediate stock solutions (5 mM and 1 mM) were diluted with milli-Q water. The test solutions were diluted from these intermediate stock solutions with 1xPBS according to table 4.

Table 4. The test solutions were prepared with 10 different concentrations. The concentrations are reduced by half when equal amounts of test solution and melanin suspension are combined.

prepared test solution concentration (µM)	**exposure concentration during the experiment (µM)**
1000	500
500	250
200	100
100	50
50	25
20	10
11.6	5.8
4	2
2	1
1	0,5

Sepia melanin suspension 2 mg/ml and drug solution at appropriate concentration were combined, 75 µl equally. Every compound and concentration were treated individually as 3–6 replicates. The incubation was conducted as described before. The control samples without melanin were prepared my mixing equal volume (75 µl) of test solution and 1xPBS in 3−6 replicates. The controls were incubated simultaneously with melanin samples.

After the incubation, the sepia melanin was spun down as earlier. Supernatant (130 µl) from each sample and control was collected to 96-well plate. The plates were sealed tightly, stored at –20 °C and eventually analyzed by a liquid chromatography-mass spectrometry (LC-MS/MS, Waters Xevo TQ-S triple quadrupole mass spectrometer).

The melanin binding data was evaluated with Sip's isotherm

$$B = \frac{B_{max}[L]^n}{K_d^n + [L]^n} \quad (4)$$

where B is the amount of bound drug, B_{max} is maximum binding capacity (nmol/mg), [L] is free drug concentration (µM), K_d is dissociation constant (µM), and n is heterogeneity index.

7 Results

7.1 Testing of melanin for drug binding studies

Originally the binding studies were planned to be performed with synthetic melanin, and it was used in the first experiments. However, this type of melanin caused some issues by leaving the supernatant brown after incubation and centrifugation (figure 6). This indicates that some melanin is left in the supernatant. This occurrence may lead to misleading results. Additionally, synthetic melanin formed aggregates so large they clogged the pipette tips and needed to be dispersed by sonication.

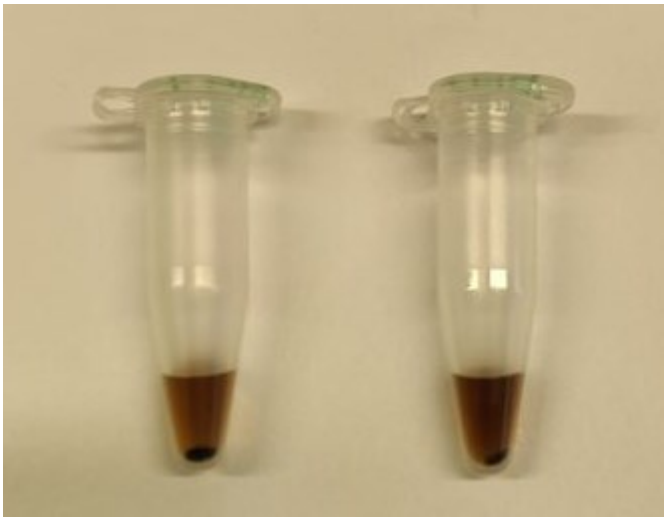

Figure 6. Synthetic melanin was incubated for 20 hours at 37 °C during binding experiment. Melanin was spun down and forms a black pellet at the bottom of the tube. The supernatant is dark brown indicating the presence of melanin in the supernatant.

A series of samples of synthetic melanin (2 mg/ml) was prepared and treated according to table 3 in order to find a way to get a clear and colorless supernatant. As sonication is needed to disperse the aggregates and make the melanin suspension more homogenic, its effects on color of the supernatant were tested. Different techniques (waterbath sonicator, probe sonicator) were compared. Supernatants were discarded and the pellets resuspended to test if the soluble component of synthetic melanin can be washed away.

As a first step, samples 1, 2 and 5 were sonicated. Figure 7 shows, that, after centrifugation, these samples are slightly darker than samples 3 and 4, which were incubated at room temperature. No difference between waterbath sonicator (samples 1 and 2) and probe sonicator (sample 5) could be seen by visual inspection.

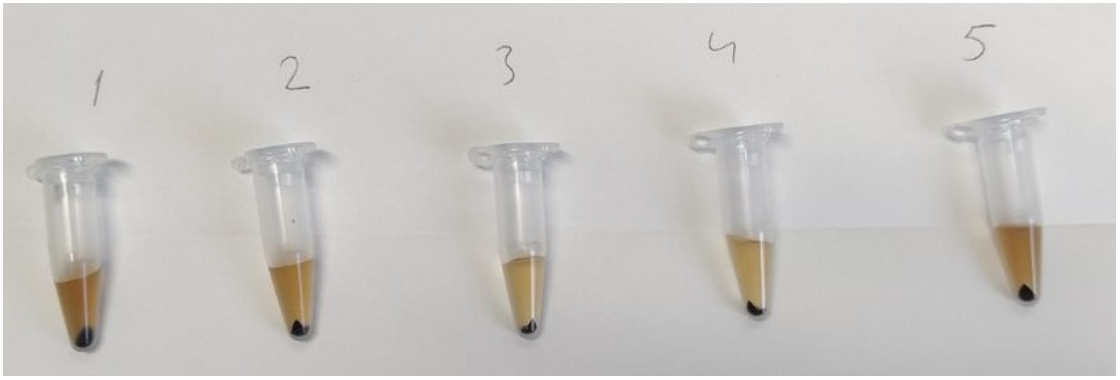

Figure 7. Samples 1 and 2 were treated with waterbath sonicator at 37 °C and sample 5 with probe sonicator. Samples 3 and 4 did not receive treatment with sonicator.

The brown supernatants were discharged, and the melanin pellets were resuspended with 1XPBS. Next, samples 1 and 4 were sonicated and samples 2, 3 and 5 were incubated at room temperature. Finally, all samples were centrifuged for supernatant color inspection (figure 8). Sonicated samples 1 and 4 are darker than incubated samples 2, 3 and 5. All samples, except sample 4, were now more light-colored than they were before PBS-washing and resuspension.

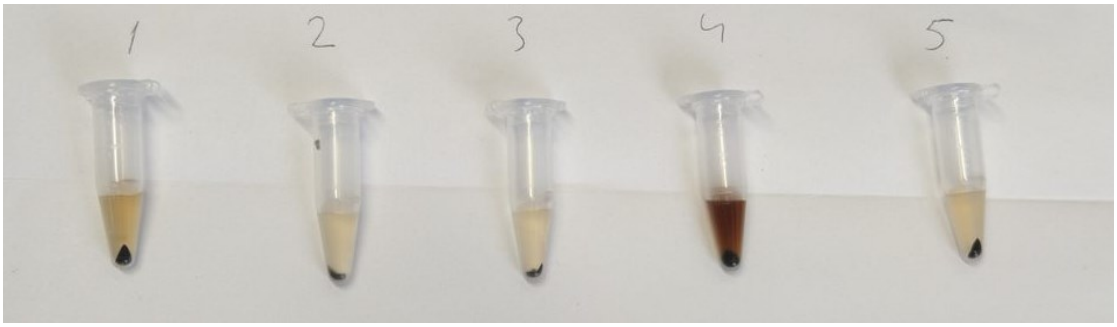

Figure 8 After removing the brown supernatant, remaining melanin pellets were resuspended at 1xPBS. Samples 1 and 4 were sonicated with waterbath sonicator, rest of the samples were incubated at room temperature without sonicating.

Despite the samples were clearer than in the beginning, they were still darker than wished. In addition, extra steps with sonication and PBS washing would be laborious and time consuming.

Therefore, sepia melanin was decided to be tested next. This kind of melanin formed visually smooth suspension immediately without any sonication step. Additionally, sepia melanin left the supernatant almost colorless after 20-hour incubation (37 °C) and centrifuging, as seen in figure 9.

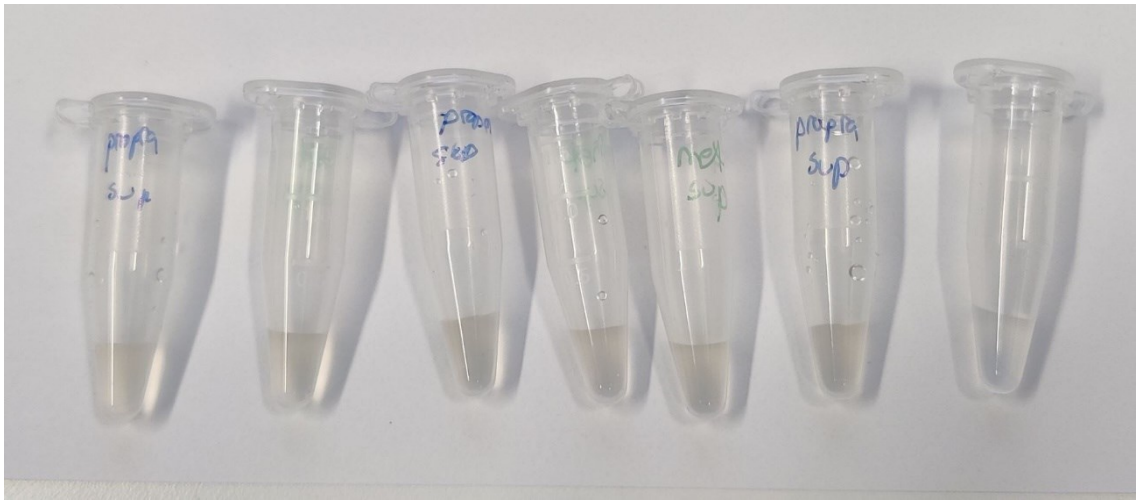

Figure 9 A binding experiment was run with sepia melanin. Supernatants were separated from melanin pellets after incubation and centrifuging. Supernatant is nearly colorless. The tube far right contains pure water for color comparison.

7.2 Melanin binding of propranolol and metformin

Two drugs, propranolol and metformin, with different melanin binding properties were chosen for first melanin binding studies. Propranolol is known to be an intermediate/high melanin binder (Pelkonen et al. 2017 a, Rimpelä et al. 2018 b) and according to unpublished data metformin can be classified as a low binder. Synthetic melanin (1 mg/ml) was used in the first two experiments, and sepia melanin in three latter experiments.

The melanin binding of propranolol is 2.6-fold higher compared to the melanin binding of metformin, regardless of the type of the melanin (figure 10). Interestingly, both drugs are bound to synthetic melanin at 1.5 times higher extent than they are bound to sepia melanin.
72 % of propranolol was bound to synthetic melanin and 47 % to sepia melanin. The respective binding fractions for metformin were 27 % and 18 %.

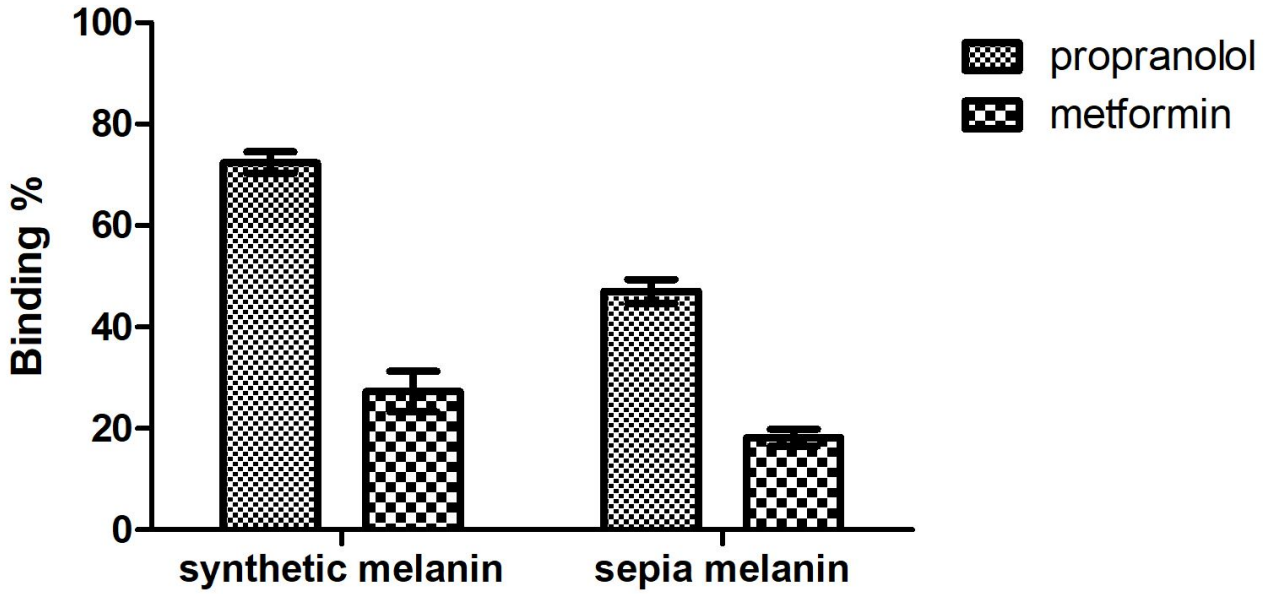

Figure 10. Radiolabeled propranolol and metformin (1 µM) were incubated with synthetic melanin and sepia melanin (1 mg/ml). The melanin binding values were calculated as a ratio of CPM in the melanin pellet and CPM in the whole sample (melanin pellet + supernatant).

Recoveries were calculated by comparing the CPM in the whole sample to CPM in control sample (table 5). Recoveries were lower in propranolol samples than in metformin samples. Recoveries were closer to 100 % in sepia melanin samples than in synthetic melanin samples.

Table 5. Recoveries (average ± SD) of melanin binding experiments. The values vary depending on the type of melanin used.

compound	**synthetic melanin**	**sepia melanin**
propranolol	49 ±6,9 %	86 ±2,2 %
metformin	110 ±45,4 %	102± 9,3 %

Sepia melanin was also noticed to form visibly lighter suspension than synthetic melanin at equal concentrations (figure 11a). To explore the different behaviors of these two types of melanin, the standard curves at equal concentrations (0.0–1.0 mg/ml) were prepared from synthetic and sepia melanin (figure 11b). Absorbances were measured at 595 nm. Synthetic

melanin forms a steeper line than sepia melanin forms: its slope is 5.6-fold compared to the slope of the line formed with sepia melanin. This suggests that sepia melanin contains only 18 % melanin, rest of the mass being impurities.

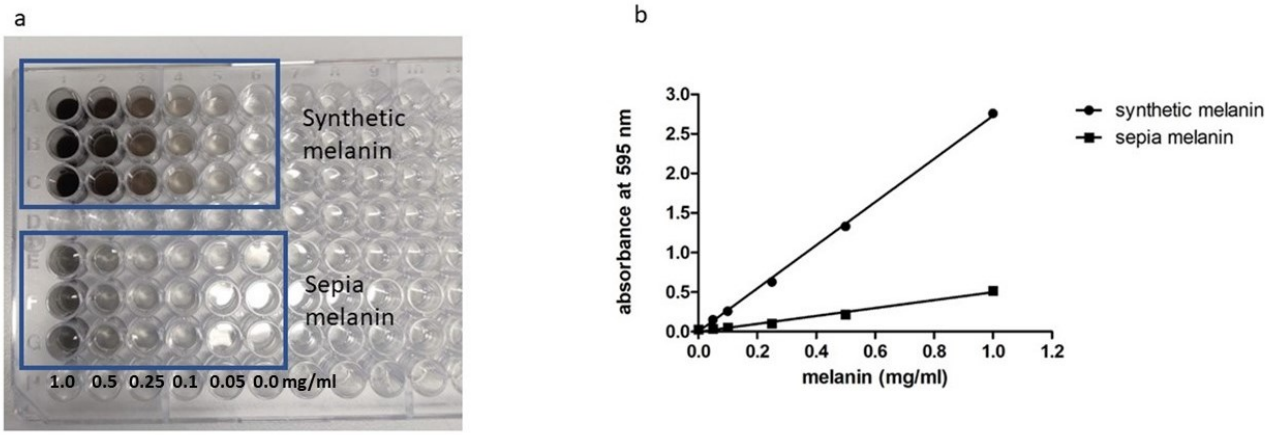

Figure 11. a) Synthetic melanin and sepia melanin standards were prepared as triplicates at concentration range 0.0–1.0 mg/ml. **b**) The absorbances were measured at 595 nm and standard curves were formed. Synthetic melanin has 5.6 times steeper line than sepia melanin.

7.3 Cell uptake studies with pigmented ARPE-19 cells

The ARPE-19 -cells were re-pigmented at pigmentation rates 0, 10, 30 and 100 % as described by Hellinen et al. (2019). Visual inspection with light microscope revealed, that cells of 100 % pigmentation were detached from the bottom of the wells. This incident happened every time when cells were re-pigmented and indicates that the cells did not tolerate high melanosome exposure. Therefore, no melanosome or drug uptake data could be obtained with 100 % pigmentation rate. However, re-pigmentation with other pigmentation rates succeeded: melanosomes were taken up by ARPE-19 -cells (figure 12), and the cells seemed to be thriving when inspected with light microscope. These cells were used in further uptake studies.

Cells of planned pigmentation rates 30 % and 10 % were incubated with 20 µg and 7 µg melanosomes. The results of measurements of absorbances show, that melanin amount taken up by 30 % cells was 14.6 µg, and by 10 % cells it was 8.3 µg. Thus, pigmentation recoveries were 73 % and 119 %, respectively.

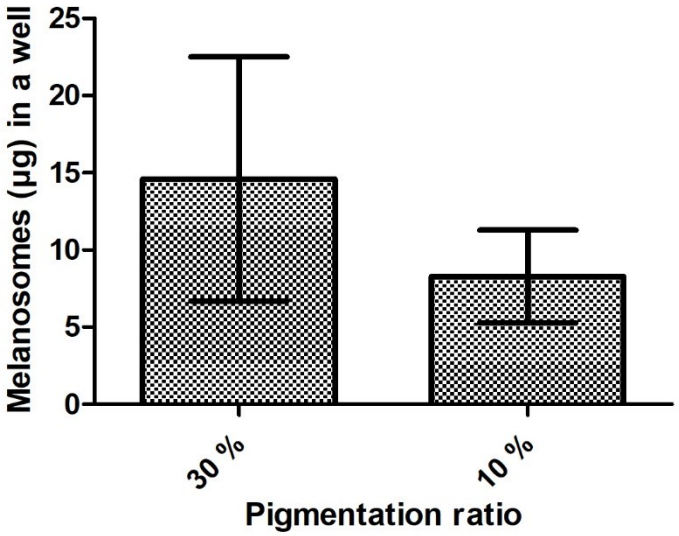

Figure 12. ARPE-19 -cells were re-pigmented with isolated porcine melanosomes and amounts of pigment (µg per well) taken up by cells were determined spectrophotometrically at 595 nm. Three pigmentation experiments were run. 30 % cells (n=17) were incubated with 20 µg melanin and 10 % cells (n=17) with 7 µg melanin. The bars illustrate the amount of melanin taken up by the cells, and error bars show standard deviations.

Cell uptake studies were conducted with 5 µM propranolol and 5 µM metformin. 4 % of propranolol and 0.26 % of metformin was taken up by non-pigmented ARPE-19 cells (figure 13).

Propranolol is an intermediate/high melanin binder (Pelkonen et al. 2017 a, Rimpelä et al. 2018 b), and cell pigmentation increases its uptake greatly: 7.3 % and 81.5 % of it was taken up by 30 % and 10 % cells. Thus, the cellular uptake to pigmented ARPE-19mel cells is 18–20 times higher than uptake to ARPE-19 cells. Melanosomal drug uptake of propranolol was 69.1 % and 77.2 % in 30 % and 10 % cells, respectively. These values are near to the propranolol's binding to synthetic melanin (72 %).

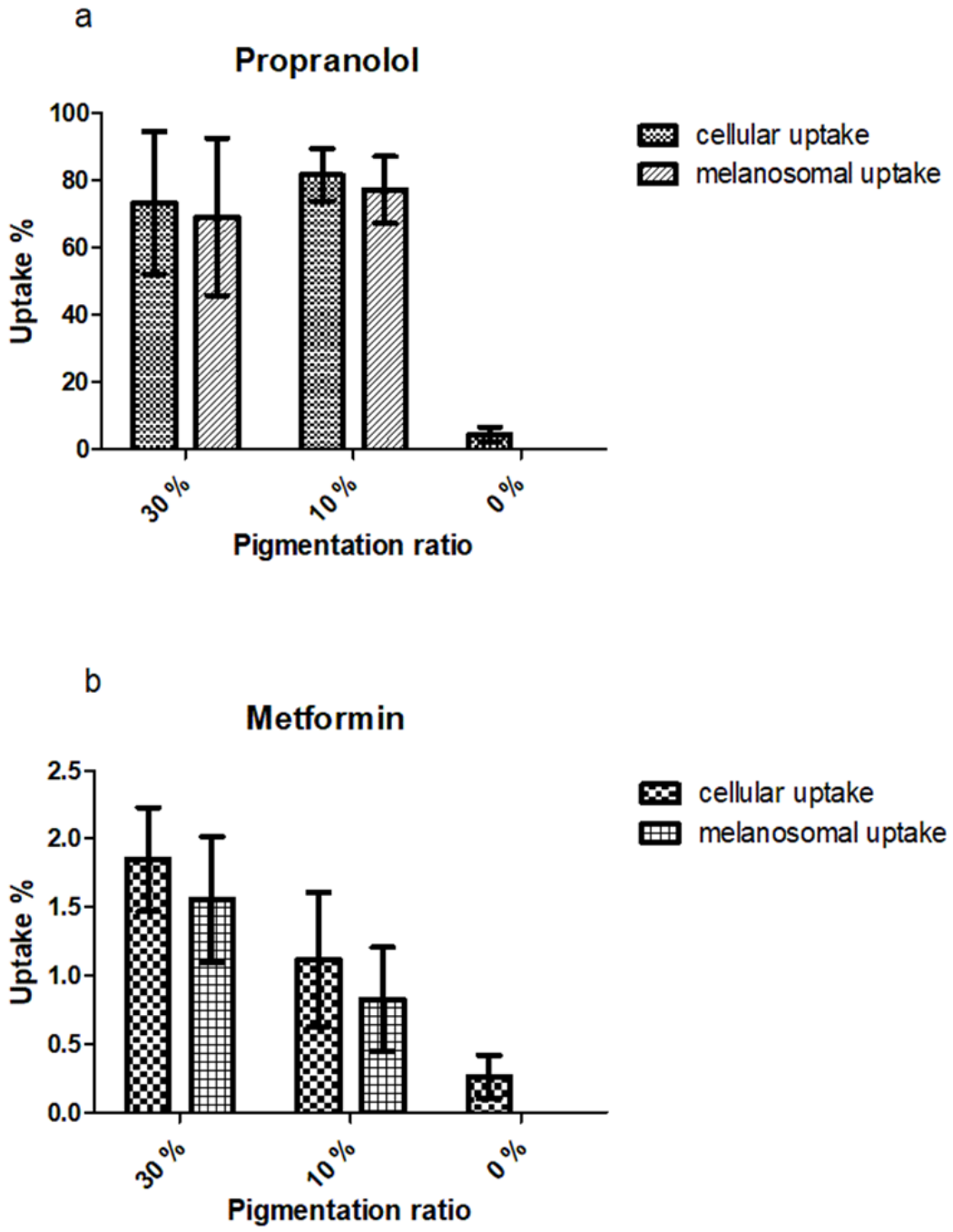

Figure 13. The drugs were incubated with ARPE-19 cells of different pigmentation ratios in three different experiments. Cellular uptake was calculated: cellular uptake (%) = (CPMcell / CPMcell+medium) *100 %. Melanosomal uptake was acquired: melanosomal uptake (%) = Cellular uptakeARPE-19 (%) – Cellular uptakeARPE-19mel (%). Bars illustrate taken up drug amounts on average, error bars standard deviations. a) Cellular and melanosomal uptake of propranolol. b) Cellular and melanosomal uptake of metformin.

Metformin, in turn, is known to be a low melanin binder (unpublished data). However, pigmentation increases its cellular uptake. 1.12 % was taken up by 10 % cells and 1.85 % by 30 % cells. These uptake fractions are 4 and 7 times higher than the uptake to non-pigmented cells. Melanosomal metformin uptake was 0.83 % in 10 % cells and 1.56 % in 30 % cells. It increases in nearly same ratio as cellular melanosome uptake increases. The increase is 1.9-fold in melanosomal drug uptake while it is 1,8-fold in cellular melanosome uptake. However, melanosomal metformin uptake is lower than metformin binding to synthetic (27 %) and sepia melanin (18 %).

Recoveries were calculated and collected to table 6. Recovery values decrease as cell pigmentation ratios increase. Propranolol has lower recovery values in re-pigmented cells than metformin does, but no different in non-pigmented cells is seen.

Table 6. Recoveries (average ±SD) of cell uptake experiments. Recoveries of different pigmentation ratios are specified. Higher melanosome content reduces the recovery.

	30 %	**10 %**	**0 %**
propranolol	72 ±10,3 %	79 ±6,0 %	92 ±4,2
metformin	86 ± 4,7 %	89 ± 3,7 %	92 ± 3,5

7.4 Melanin binding of ganciclovir, voriconazole, quinidine and ciprofloxacin

The sepia melanin binding was studied at concentration range 0.5 – 500 µM. The binding data is collected to figure 14. Ganciclovir and voriconazole are low binders (Pelkonen et al. 2017 a). Their binding is staying at low levels: less than 30 % of ganciclovir and less than 20 % of voriconazole is bound. No saturation can be seen.

Quinidine and ciprofloxacin, in turn, are high binders (Pelkonen et al. 2017 a). Nearly 100 % of both drugs is bound to melanin at low exposure concentrations. As the exposure concentration increases, the melanin binding of quinidine starts to saturate, and the bound fraction of the

inserted drug decreases to 40 %. Ciprofloxacin does not show saturation, as the drug binding fractions stay over 80 % even at the highest concentration of drug exposure.

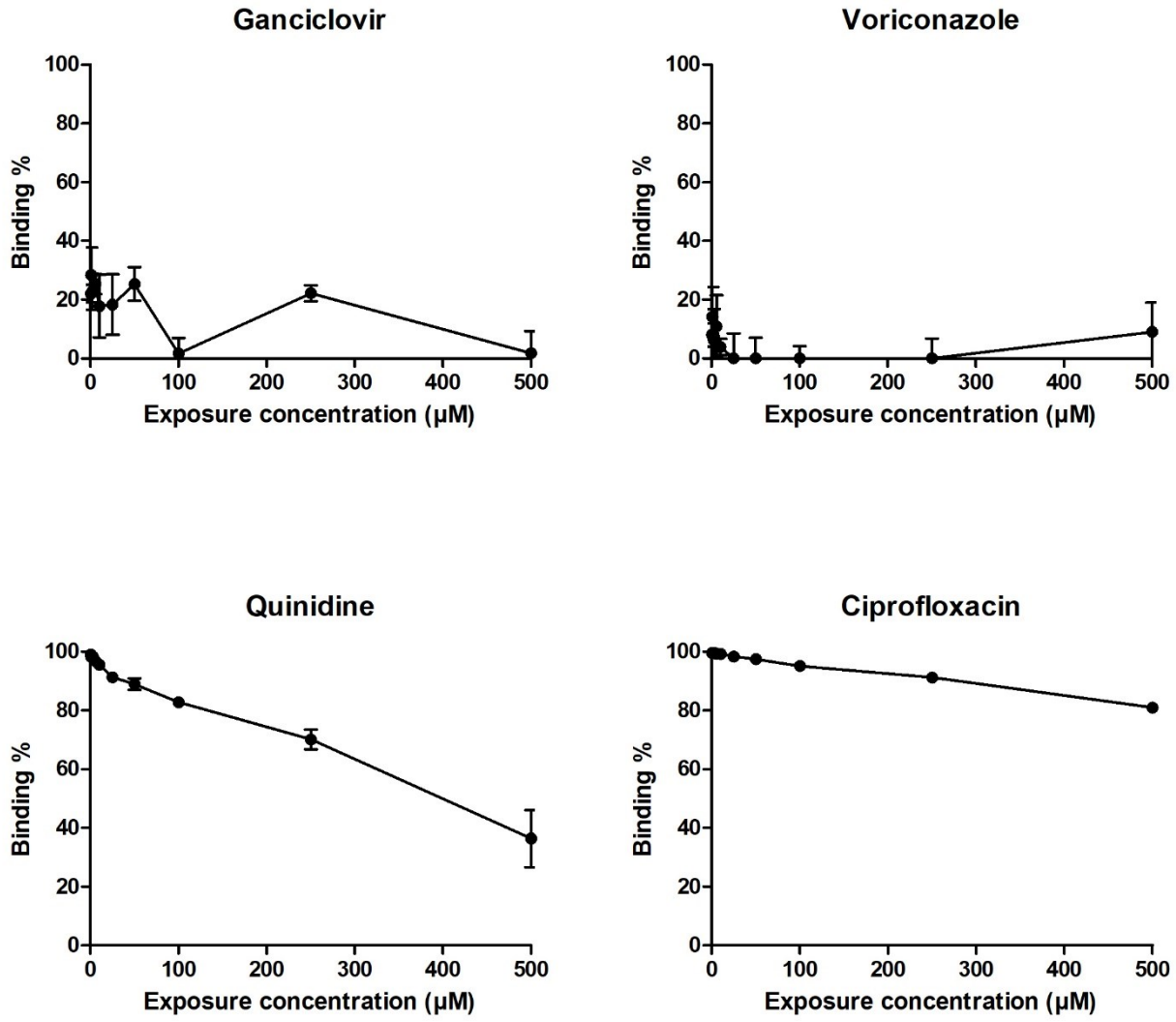

Figure 14. Sepia melanin binding of ganciclovir, voriconazole, quinidine and ciprofloxacin was studied on large range of concentrations (0,5 – 500 µM) as 3-6 replicates, error bars illustrate standard deviations. The samples were taken from supernatants and melanin binding was calculated: melanin binding (%) = (control – sample)/control *100 %.

Melanin binding of drugs can be predicted with Sip's isotherm (equation 4) (Manzanares et al. 2016). The model was fitted to the experimental data in direct plot (figure 15). Overall, the fit is better with high melanin binders than low melanin binders. Melanin binding is predicted well at

low (less than 50) free drug concentrations, but at higher free drug concentrations predicted values (bound nmol/mg) differ from experimental values.

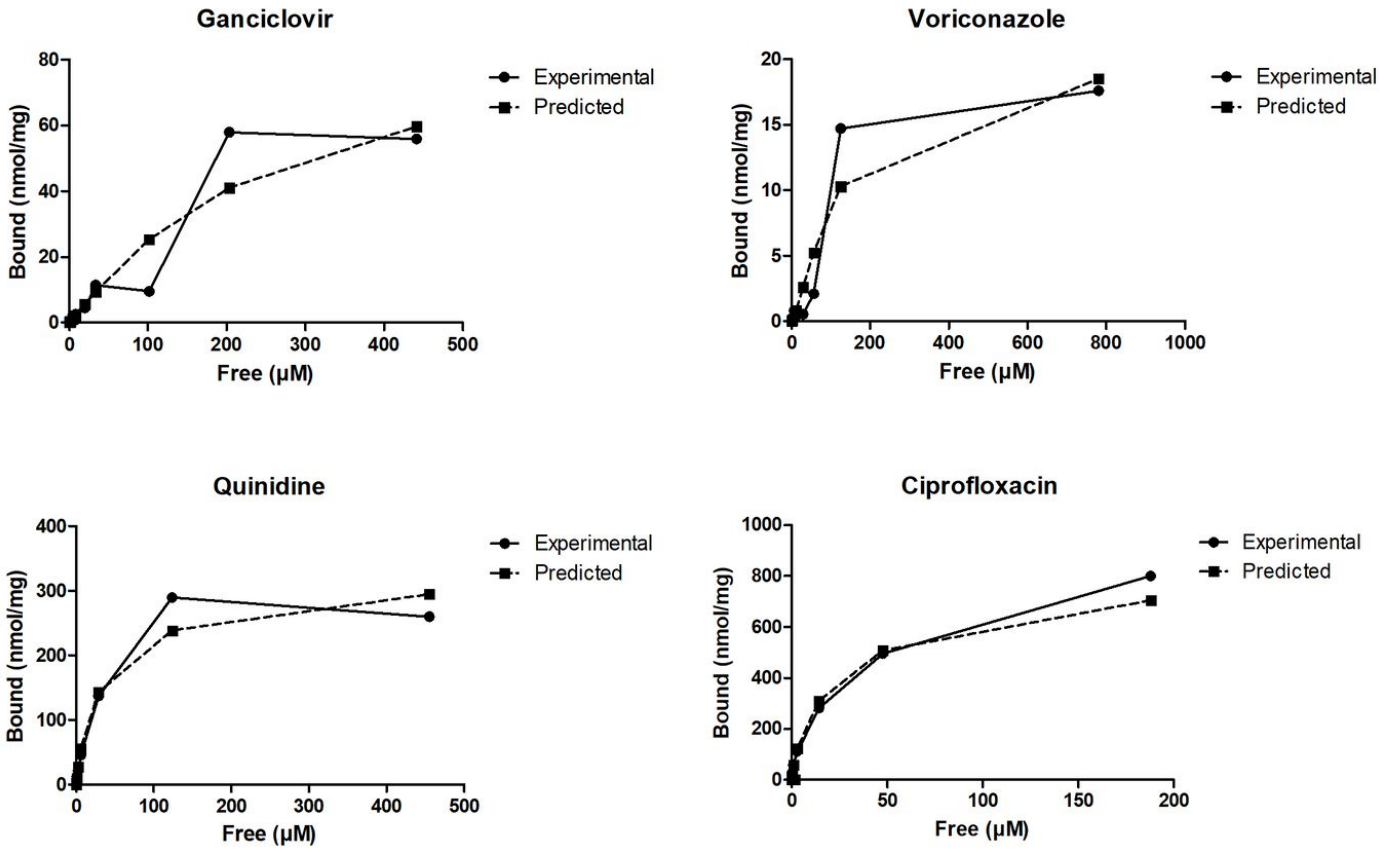

Figure 15. Experimental (solid lines) and predicted (dashed lines) binding data of ganciclovir, voriconazole, quinidine and ciprofloxacin in direct plots.

Table 7 collects the binding parameters estimated with Sip's isotherm. B_{max} of high melanin binders are remarkably higher than respective values of low binders. The difference between the highest binder ciprofloxacin (B_{max} = 900 nmol/mg) and the lowest binder voriconazole (B_{max} = 19.9 nmol/mg) is 45-fold. High melanin binders have lower dissociation constants (K_d) than low melanin binders have. The difference is approximately 7-fold at its highest. Heterogeneity indexes (n) are below 1 for high melanin binders and above 1 for low melanin binders. $k = B_{max}/K_d^n$ can be determined with low uncertainty (Manzanares et al. 2016). This parameter can also be used to estimate and compare binding affinities: the higher the value of k is, the higher the binding affinity is. k for high melanin binders is notable greater than for low melanin binders.

Table 7. Estimates ±SD for binding parameters were acquired with Sip's isotherm.

	B_{max} (nmol/mg)	**K_d (µmol/L)**	**n**	**k (Bmax/Kd^n)**
Voriconazole	19.9 ± 5.7	119.5 ± 69.7	1.4 ± 0.38	0.01
Ganciclovir	90.9 ± 56.6	243.9 ± 288.6	1.1 ± 0.28	0.21
Quinidine	336.5 ± 48.7	41.3 ± 20.6	0.8 ± 0.10	17.1
Ciprofloxacin	900.0 ± 116.4	33.7 ± 13.9	0.75 ± 0.06	64.3

Based on the binding data analyzed with Sip's isotherm, the drugs can be ordered from the lowest melanin binder to the highest binder: voriconazole, ganciclovir, quinidine, ciprofloxacin.

8 Discussion

Synthetic melanin and sepia melanin behaved differently during the experiments. Synthetic melanin left the supernatant brown after centrifugation whereas sepia melanin left the supernatant clear. Synthetic melanin had higher absorbances than sepia melanin, suggesting that melanin content in synthetic melanin is higher than in sepia melanin at equal concentrations. Propranolol and metformin were also bound in a higher extent to synthetic melanin than to sepia melanin. DHI/DHICA ratio determines multiple properties of melanin, e.g., particle size and light absorption and compound binding properties (García-Borrón and Olivares Sánchez 2011, Rimpelä et al. 2018 a). Synthetic melanin is consistent mainly of DHI, but in natural melanins, including sepia melanin, proportion of DHICA exceeds that of DHI (Rimpelä et al. 2018 a). Many differences seen between behaviors of synthetic melanin and sepia melanin are due partially to different ratios of DHI and DHICA.

Depending on suspension medium, synthetic melanin has been shown to have variable particle size distributions (Koeberle et al. 2003, Jakubiak et al. 2019). When suspended at deionized water, synthetic melanin forms particles with sizes distributed unimodally between 0.2 and 19 µm, mode being at 0.8 µm (Koeberle et al. 2003). At PBS with pH 7.4, particle size distribution (PSD) is bimodal ranging between 0.2 and 6 µm, with modes at 0.8 and 1.9 µm. Jakubiak et al. (2019a) analyzed particle sizes at PBS with pH 6.5, and synthetic melanin showed large and trimodal PSD between 1-1000 µm, medians being at 9, 89 and 452 µm. However, suspension medium does not seem to influence remarkably on the particle sizes of sepia melanin (Koeberle et al. 2003, Jakubiak et al. 2019). Particle sizes are distributed unimodally, with a mode at 12.4 µm (Koeberle et al. 2003) or median at 15 µm (Jakubiak et al. 2019). It is possible that the smallest particles of synthetic melanin are left to the supernatant after centrifugation giving it a brown color, whereas larger particles of synthetic melanin and sepia melanin form the melanin pellet to the bottom of the tube. However, pH was not controlled, and particle sizes were not studied in these experiments.

Absorbances of synthetic melanin at 595 nm wavelength were higher than respective absorbances of sepia melanin. The absorbance data suggests that sepia melanin contains only 18 % melanin. Sepia melanin is known to contain impurities such as proteins and metal ions, which lower the true melanin concentration in sepia melanin suspension prepared by weighing (Pezzella et al. 1997). In addition to impurities, certain properties of melanin can affect its light absorbance (Haywood et al. 2006, Li et al. 2015, Ju et al. 2018). Larger melanin particles contribute to higher light absorbances than smaller particles (Li et al. 2015, Ju et al. 2018). Haywood et al. (2006) have shown with synthetic melanin, that state of oxidation as well effects on absorbance spectrum of melanin: oxidation increases absorbance at lower wavelengths but decreases it at higher wavelengths.

Propranolol and metformin were radiolabeled (H3 and C14, respectively), and samples were analyzed with scintillation counter. Melanin's effect on recovery values was seen clearly, especially during cell experiments: increasing melanin amounts weakened the recoveries. Melanin absorbs electromagnetic radiation in a wide range of frequencies, also in gamma ray area (Casadevall et al. 2017). As discussed earlier, synthetic melanin and sepia melanin are very different, hence they effect on recoveries differently. The impact was stronger on recoveries of propranolol samples than metformin samples. Propranolol is a high melanin binder and most of the detected signals are emitted from melanin sample and are weakened by it. Metformin, in turn, is a low melanin binder and most of the signals emitted by it are detected from supernatant sample, where melanin doesn’t weaken them.

According to unpublished data, when pigmented samples are analyzed with scintillation counter, a correction coefficient should be determined at studied pigmentation concentrations to acquire reliable results. Scintillation counter is a simple method to analyze melanin binding of radiolabeled compounds, and CPM can be measured straight from the melanin pellet, considering the correction coefficient. However, this method requires the compound to be radiolabeled. Few radiolabeled compounds are commercially available, and some compounds can be radiolabeled in house. LC-MS/MS, in turn, requires careful method optimization and sample handling, and only samples collected from supernatant can be analyzed with this

method. On the other hand, availability of compounds wished to study is not an issue as it might be with scintillation counter.

Melanin binding of six different drugs were studied. Propranolol and metformin were studied at single concentrations to estimate their binding class. The melanin binding classes are low (less than 50 %), intermediate (50–80 %) and high (more than 80 %) (Pelkonen et al. 2017 a). Ganciclovir, quinidine, voriconazole and ciprofloxacin were studied at a wide range of concentrations, and Sip's isotherm was used to estimate Bmax and Kd for them. Sip's isotherm is known to be the most suitable model for estimating melanin binding parameters, as it considers heterogeneity and different binding energies on the surface of melanin particles (Manzanares et al. 2016). Propranolol, quinidine and ciprofloxacin showed intermediate/high binding to melanin, whereas metformin, ganciclovir and voriconazole acted as low melanin binders. In general, lipophilic and basic compounds are known to be good melanin binders (Leblanc et al. 1998). Jakubiak et al. (2018) have developed a model to predict the melanin binding of drugs. According to this model, the most favorable range of basic pKa of compound is 5.7–9.4, and lipophilicity and aromatic rings contribute to melanin binding. Propranolol, quinidine and ciprofloxacin meet this criteria (Drugbank 2023). Propranolol has been reported to be intermediate/high melanin binder, quinidine and ciprofloxacin are known to be high melanin binders (Pelkonen et al. 2017a, Rimpelä et al. 2018b, Hellinen et al. 2019). Despite lipophilicity and aromatic rings, voriconazole is only weakly basic. Ganciclovir also has low basic pKa and in addition to this, it is hydrophilic. Metformin is also hydrophilic, and its strong basic nature increases this feature by quick ionization (Jakubiak et al. 2018). Additionally, metformin does not have aromatic rings in its structure (Drugbank 2023). Voriconazole, ganciclovir and metformin have been reported to be weak melanin binders (unpublished data, Pelkonen et al. 2017a) It seems that Sip's isotherm works better for high melanin binders (voriconazole and ciprofloxacin) than low binders (ganciclovir, quinidine). Especially ganciclovir showed great variation in estimates, and heterogeneity index n exceeds 1 in both cases of low binders, whilst it is supposed to stay below 1. However, parameter k showed clearly, that ciprofloxacin is the highest and voriconazole the lowest melanin binder.

Propranolol and metformin were bound to synthetic melanin in a higher extent than to sepia melanin. These results are opposite to the results by Koeberle et al. (2003) and Jakubiak et al. (2019), who have found sepia melanin to be stronger drug binder than synthetic melanin. However, experimental setups (e.g., melanin processing, studied drugs, pH, analytics) are different in these studies, and thus the results are not fully comparable. Most importantly, the results of spectrophotometric studies suggest that melanin concentration in sepia melanin suspension is lower than in suspension prepared of synthetic melanin. Melanin concentration has an impact on the results of binding experiments: lower melanin amount can bind lower proportion of the drug than higher melanin amount (Pelkonen et al. 2017a). However, it is notable, that melanin bound fraction of the drug doesn't necessarily grow at the same rate than melanin concentration. Direct conclusions about drug binding to sepia melanin vs synthetic melanin should not be done based on the data of this thesis. Determining B_{max} and K_d, for example with Sip's isotherm, in both synthetic melanin and sepia melanin would provide more accurate information about the different binding properties of these two melanin types.

The pigmentation of 10 % and 30 % ARPE-19mel cells succeeded, and pigmentation recoveries were similar to results by Hellinen et al. (2019). Interestingly, cells of 100 % pigmentation, incubated with 68 µg melanin per well, were lost. Cells are expected to tolerate this melanin amount, as Hellinen et al. (2019) have managed to pigment the cells with the dose as high as 204 µg melanin per well. However, it is notable that Hellinen et al. quantified melanin contents of melanosomes with porcine melanin as standard, but in these studies standards were prepared of synthetic melanin. Hence, the true mass of melanin inserted to cells may differ from the study by Hellinen et al., but no conclusions about toxic melanin amounts can be made without further studies. The proportion of PBS in cell growth medium varied between 5 and 10 % in the wells of 100 % cells, and this proportion is not expected to be toxic. Thus, the reasons for the loss of 100 % cells remain unclear.

The accumulation of propranolol was greatly higher to pigmented cells than to non-pigmented cells. No difference between pigmentation rates of 10 % and 30 % was seen. Propranolol is a high melanin binder, and its high lipophilicity (logP 3,48, Drugbank 2023) and low molecular

weight enables it to penetrate across cell and melanosome membranes by passive diffusion and to be bound to melanin. Compared to results of Hellinen et al. (2019), melanosomal drug uptake was at the same level in 30 % cells, but at higher level in 10 % cells.

Cellular accumulation of metformin was low but slightly increased by cell pigmentation. Melanosomal uptake was increased at the same rate as pigmentation increased between 10 % and 30 % cells. Metformin is a lipophobic compound (logP -2,6, Drugbank 2023) and it can't penetrate across the membranes by passive diffusion. Metformin is known to be a substrate of plasma membrane monoamine transporter (PMAT), organic cation transporter 1 (OCT1), OCT2, OCT3, multidrug and toxin extrusion 1 (MATE1) and MATE2-K (Gong et al. 2012). ARPE-19 are found to express MATE1, multidrug resistance associated protein 1 (MRP1), MRP4, MRP5, MRP7, glucose transporter 1 (GLUT1), 4F2hc, taurine transporter (TAUT), cationic amino acid transporter 1 (CAT1), large neutral amino acid transporter 1 (LAT1), monocarboxylate transporter 1 (MCT1), MCT4, proton-coupled folate transporter (PCFT) and Na+/K+ ATPase (Pelkonen et al. 2017b). Metformin is taken up to the cells by MATE1, which transports its substrates in the direction opposite to H+ gradient, and functions as an uptake transporter in physiological pH (Terada et al. 2006).

9 Conclusions

Melanin binding of six different drugs was studied. Propranolol, quinidine and ciprofloxacin are high melanin binders; metformin, ganciclovir and voriconazole low melanin binders. Sip's isotherm was seen to be suitable for estimating B_{max} and K_d for high melanin binders. Four of the drugs can be ranked based on their melanin binding: voriconazole < ganciclovir < quinidine < ciprofloxacin. Different behavior of synthetic melanin and sepia melanin was seen clearly. Melanin type and concentration have an impact on the results of drug binding studies. Melanin's ability to absorb electromagnetic radiation needs to be considered when scintillation counter is used as analytics. Additionally, re-pigmentation of ARPE-19 cells increases drug accumulation to the cells.

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