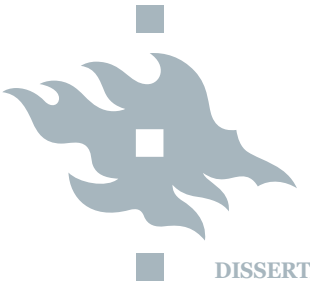

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Ocular Pharmacokinetic Effects of Drug Binding to Melanin Pigment and the Vitreous Humor

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OCULAR PHARMACOKINETIC EFFECTS OF DRUG BINDING TO MELANIN PIGMENT AND THE VITREOUS HUMOR

Anna-Kaisa Rimpelä

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in lecture room 1, Metsätalo (Unioninkatu 40), on 16 June 2018, at 12 noon.

Helsinki 2018

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ABSTRACT

Diseases of the posterior segment of the eye, such as age-related macular degeneration and diabetic retinopathy, are the leading causes of blindness in the developed world. Drug delivery to the posterior eye tissues, including the retina and choroid, is accomplished by frequent intravitreal injections, which are expensive and burdensome to the health care providers and patients, and may cause harmful side effects. Therefore, sustained and less invasive delivery strategies are vitally needed to improve the treatment. Melanin pigment is found at high concentrations in ocular tissues, and many clinical drugs bind to it. Targeting the pigment to obtain sustained drug action in ocular tissues could be a feasible approach for improving ocular drug therapy. Therefore, this thesis project aimed to study the effects of pigment binding on pharmacokinetics in the eye with *in vitro*, *in vivo* and *in silico* methods. In addition, we investigated drug binding to the vitreous humor, as it may participate in modulating ocular pharmacokinetics and has been studied only scarcely.

Melanin is a negatively charged, hydrophobic polymer, and it is expected to bind all basic and lipophilic drugs to some extent. Melanin is located in intracellular melanosomes, which are cell organelles surrounded by a lipid membrane, and are expected to have an acidic intraluminal pH. Due to the location of melanin, cellular and physiological factors act in concert with the binding to the melanin polymer, to impact ocular pharmacokinetics of melanin-binding drugs. We investigated pigment binding and related cellular and physiological factors with seven small molecule drug or drug-like compounds: chloroquine, timolol, nadolol, propranolol, methotrexate, 5(6)-carboxy-2′,7′-dichlorofluorescein (CDCF) and diclofenac. A pH-dependent binding (pH 5.0 vs. 7.4) to isolated porcine ocular melanin was observed, mainly with the acidic compounds that are less negatively charged and bind better at pH 5.0. Therefore, pH plays an important role in drug-melanin binding in the case of acidic drugs, but is less important in the case of basic drugs. The binding parameters, maximum binding capacity and affinity, were reliably calculated with the Sips binding isotherm instead of the commonly used Langmuir isotherms. The Sips isotherm is in line with the heterogeneous nature of the melanin surface to where the drugs bind, and, therefore, is better suitable for parameter analysis. Cellular uptake and intracellular binding in pigmented retinal pigment epithelial cells were shown to correlate with melanin binding, but other factors, such as lipophilicity of the drug, also need to be taken into account. Pharmacokinetic simulations of the retention of drugs in pigmented posterior segment tissues demonstrated that low drug permeability in the plasma and melanosomal membranes and the entrapment of positively charged drugs in the melanosome increases melanin binding related retention in these tissues. In addition, both the intracellular binding

experiments with pigmented cells and the pharmacokinetic simulations showed that only a small fraction (~0.01%) of the highest binding drug of this study, chloroquine, is in the free form inside the cells. The free form of the drug elicits the drug action (beneficial or harmful), and it is, therefore, important to differentiate between the free and total drug inside the cells when assessing drug response. Furthermore, we demonstrated the distribution and retention of ^{123}I-chloroquine in the eyes of pigmented but not albino rats after intravenous administration, establishing the use of single photon emission computed tomography/computed tomography imaging in monitoring melanin binding related kinetics *in vivo*. All in all, in the variety of experiments performed, melanin binding was shown to have a major impact on ocular pharmacokinetics in pigmented tissues. In addition to investigating melanin binding, drug binding to the vitreous humor was studied with a cassette of 35 clinical small molecule drugs. The binding was rather low and vitreal binding was concluded to have only a modest effect on ocular pharmacokinetics.

In conclusion, this thesis project generated important information of the extensive pharmacokinetic impact of drug-melanin binding in ocular tissues. In addition, we demonstrated the moderate pharmacokinetic impact of vitreal drug binding, which is not comparable to melanin binding. The computational models developed on melanin binding and its pharmacokinetic implications can be used in drug discovery and development. The *in vitro* methods can also be implemented to the industry scale drug development process. The results obtained support the feasibility of using melanin targeting to attain sustained action in pigmented ocular tissues, but more research into the approach is needed before it can be employed in practice.

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Anna-Kaisa Rimpelä
Helsinki, May 2018

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Manzanares J, **Rimpelä A-K**, Urtti A: Interpretation of ocular melanin drug binding assays. Alternatives to the model of multiple classes of independent sites. Mol Pharmaceutics 13: 1251-1257, 2016

II **Rimpelä A-K**, Schmitt M, Latonen S, Hagström M, Antopolsky M, Manzanares J, Kidron H, Urtti A: Drug distribution to retinal pigment epithelium: studies on melanin binding, cellular kinetics, and single photon emission computed tomography/computed tomography imaging. Mol Pharmaceutics 13: 2977-2986, 2016

III **Rimpelä A-K**, Reinisalo M, Hellinen L, Grazhdankin E, Kidron H, Urtti A, del Amo E: Implications of melanin binding in ocular drug delivery. Adv Drug Deliv Rev (*In press*)

IV **Rimpelä A-K**, Hagström M, Kidron H, Urtti A: Melanin targeting for intracellular drug delivery: quantification of bound and free drug in retinal pigment epithelial cells (*Manuscript*)

V **Rimpelä A-K**, Reunanen S, Hagström M, Kidron H, Urtti A: Binding of small molecule drugs to porcine vitreous humor. Mol Pharmaceutics (*In press*)

The publications are referred to in the text by their roman numerals.

AUTHOR CONTRIBUTION

Publication I
The author designed the experiments with the co-authors. The author performed the melanin binding experiments. The author participated in writing the manuscript.

Publication II
The author designed the experiments with the co-authors. The author performed all the *in vitro* experiments (melanin binding, cell uptake and elimination, particle characterization). The author performed the cell uptake calculations. The author wrote the first draft of the manuscript and revised it with the help of the co-authors.

Publication III
The author designed and performed the pharmacokinetic simulations. The author wrote the first draft of the manuscript and revised it with the help of the co-authors.

Publication IV
The author designed the experiments with the co-authors. The author performed all the experiments and predictive calculations. The author wrote the first draft of the manuscript and revised it with the help of the co-authors.

Publication V
The author designed the experiments with the co-authors. The author supervised the *in vitro* experiments and performed the pharmacokinetic simulations. The author wrote the manuscript with the help of the co-authors.

ABBREVIATIONS

AH	aqueous humor
AMD	age-related macular degeneration
AUC	area under the concentration curve
B_{max}	maximum binding capacity
CDCF	5(6)-carboxy-2′,7′-dichlorofluorescein
DCT	dopachrome tautomerase
DHI	dihydroxyindole
DHICA	dihydroxyindole carboxylic acid
DME	diabetic macular edema
DR	diabetic retinopathy
FDA	Food and Drug Administration (United States)
HPLC	high-performance liquid chromatography
ILM	inner limiting membrane
IOP	intraocular pressure
IPE	iris pigment epithelium
IQ	indolequinone
IQCA	indolequinone carboxylic acid
K_d	dissociation constant
LC-MS/MS	liquid chromatography-tandem mass spectrometry
logD	water-octanol partition coefficient at a certain pH
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRP	multidrug resistance associated protein
MRSA	methicillin-resistant *Staphylococcus aureus*
n	heterogeneity index in the Sips binding isotherm
NIU	non-infectious uveitis
P_{app}	apparent permeability
PCE	pigmented ciliary epithelium
PD	pharmacodynamic(s)
PK	pharmacokinetic(s)
pK_a	negative logarithm of acid dissociation constant
pRPE	primary porcine retinal pigment epithelium
QSAR	quantitative structure-activity relationship
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
SPECT/CT	single photon emission computed tomography/computed tomography
UPLC	ultra-performance liquid chromatography
UV	ultra-violet
V_d	volume of distribution
wAMD	wet age-related macular degeneration

1 INTRODUCTION

Eyesight has always been considered the most important of our five senses. The fear of sight loss in people diagnosed with conditions with a prognosis of blindness can lead to severe psychological distress (De Leo et al., 1999). Visual impairment has been shown to decrease the quality of life more than many other chronic conditions (e.g. cancer, ischemic heart disease, diabetes, and depression) (Park et al., 2015) and the quality of life is lower in people with acquired than with congenital blindness (Vuletić et al., 2016). Diseases of the posterior segment of the eye, such as diabetic retinopathy (DR) and age-related macular degeneration (AMD), are the leading cause of irreversible blindness in developed countries (Wong et al., 2014). These diseases are mostly a problem of the aging population, as for example the prevalence of age-related maculopathy in the Finnish population increased from 1% in people aged 20-64 to 27% in the population aged 85 and over (Laitinen et al., 2009). Also other vision-threatening diseases of the eye, such as glaucoma and cataract, increase with age. Thus, the exponential aging of the population is likely to increase the prevalence of ocular diseases remarkably. Globally, there are expected to be 200 million AMD patients in 2020 and 290 million in 2040 (Wong et al., 2014).

There are great challenges in treating diseases of the posterior eye segment; some of the diseases do not have effective drug treatments, and drug delivery to reach the target tissues poses a challenge for the treatment and drug development. Currently, AMD is treated with anti-angiogenic agents administered through intravitreal injections given every 4-6 weeks (Tuuminen et al., 2017). In the United States alone, 4 million intravitreal injections were given in 2013, and the yearly number was expected to rise to 6-8 million by 2016 (Williams, 2014). This invasive treatment may be unpleasant, distressing and frightening for the patient, can cause harmful side effects, such as intraocular inflammation and ocular hemorrhage, requires a specialized expert to administer the drug, and is very expensive. To improve the treatment of ocular diseases, long-acting delivery strategies are needed, especially in the case of small molecule drugs (e.g. corticosteroids and antibiotics) that are cleared rapidly from the vitreous. In the case of the posterior eye segment, which is difficult to reach through topical delivery to the ocular surface, sustained delivery of the drug would decrease the frequency of invasive intravitreal administration. Targeting the posterior tissues could enable the use of less invasive administration routes, such as topical or oral delivery.

Many clinical drugs bind to melanin pigment which is found at high concentrations in ocular tissues. This thesis aimed to study the possibility of using pigment binding of drugs as an ocular targeting strategy by investigating how the binding affects the pharmacokinetics in the eye. Pigment binding is a well-known phenomenon but systematic research of the properties of the

drugs that bind and other cellular and physiological factors affecting the binding is missing. Additionally, drug binding to the vitreous humor and its impact on vitreal pharmacokinetics has been studied only scarcely. Therefore, in addition to melanin binding, we investigated drug binding to the vitreous humor. This study increases the understanding of pigment binding and vitreal binding in ocular drug treatment and provides tools for the development of new drugs.

2 REVIEW OF THE LITERATURE

2.1 PHARMACOKINETICS IN THE EYE

The eye is a well-protected organ with a variety of tissue and dynamic barriers surrounding it (Hornof et al., 2005; Urtti, 2006). In drug delivery, these barriers need to be penetrated in order to elicit the effect in the targeted tissue. Anterior tissues of the eye, the cornea, conjunctiva, aqueous humor, iris, ciliary body, and trabecular meshwork, are mainly treated by topical drug administration on the surface of the eye. This is accomplished by eye drops, ointments or gels. The posterior segment tissues, the retina, choroid, sclera and vitreous, are more difficult to reach by topical delivery, and local invasive drug delivery, such as intravitreal injection, is necessary. To develop effective drugs and drug delivery strategies for ocular diseases, a profound understanding of the ocular barriers is required.

2.1.1 BARRIERS IN OCULAR DRUG DELIVERY

Cornea and precorneal loss

Drug targets for ocular diseases are generally located in the inner parts of the eye and the drug has to penetrate at least the outermost tissue of the eye, the cornea. After the instillation of an eye drop, less than 5% of the drug generally passes the cornea to reach the aqueous humor (Maurice and Mishima, 1984). The majority of the drug is lost by the so called precorneal loss, either through overflow or nasolacrimal drainage of the tear fluid or through absorption via the conjunctiva to the systemic circulation.

In addition to the precorneal loss, the cornea itself poses a barrier for drug penetration (Figure 1, [1]). It is composed of five layers; epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium (Remington, 2012). The epithelium is five to seven cell layers thick (~50 μm), and tight junctions join the cells of the surface layer. Drugs penetrate the epithelium mainly transcellularly, benefiting the permeation of lipophilic compounds. The stroma of the cornea is hydrophilic (thickness ~500 μm), and thus favors the penetration of hydrophilic compounds. The endothelium is a single cell layer with relatively leaky tight junctional complexes and its contribution to the barrier functions of the cornea is minor. Both Bowman's layer and Descemet's membrane are collagenous layers, each 5-15 μm thick, but neither of these layers is relevant for the barrier properties of the cornea (Durairaj, 2016). In total, lipophilicity improves the drug permeation across the cornea, whereas hydrogen bonding capacity, interpreted as lowering the permeability in the hydrophilic, collagen-rich stroma, decreases the permeation (Kidron et al.,

2010). The permeability values for corneal permeability vary roughly from 5×10^{-7} cm/s for the most hydrophilic compounds towards 5×10^{-5} cm/s for the more lipophilic compounds (Kidron et al., 2010; Prausnitz and Noonan, 1998).

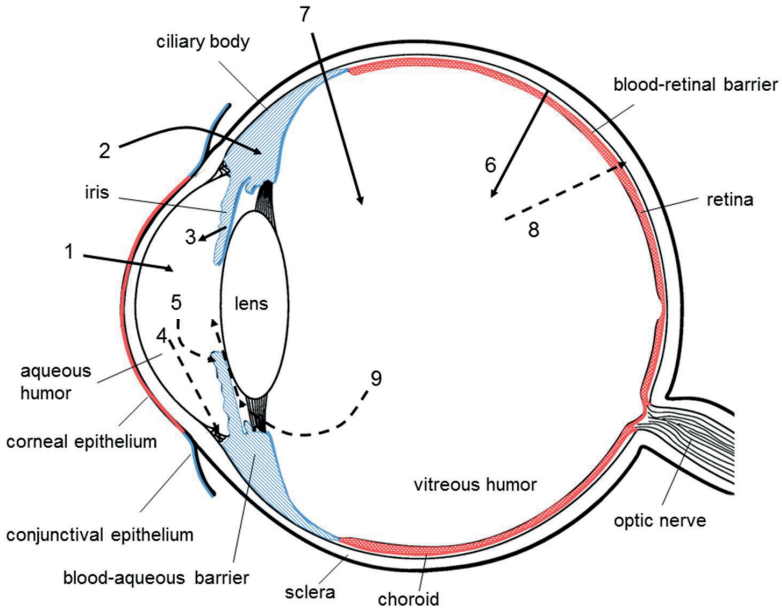

Figure 1 Structure of the eye and barriers in ocular drug delivery. The absorption and elimination of the drug takes place as follows: [1] Absorption through the cornea, [2] absorption through the non-corneal route through the conjunctiva and sclera, [3] distribution from the systemic circulation to the anterior chamber, [4] elimination through aqueous humor outflow through the Schlemm's canal and trabecular meshwork, [5] elimination through the circulation of the iris, [6] distribution from the systemic circulation to the vitreous through the blood-retina barrier, [7] intravitreal delivery, [8] elimination through the blood-retina barrier, and [9] elimination through the anterior route to the posterior and anterior chambers. Figure from Hornof et al. (2005), reprinted with permission from Elsevier.

Conjunctiva

After topical delivery, the drug can also enter the eye through the non-corneal route via the conjunctiva and sclera (Figure 1, [2]). The conjunctiva is a vascularized tissue with an apical epithelial layer and inner stroma (Remington, 2012). It can be divided into palpebral conjunctiva covering the inner surface of the eyelids and to bulbar conjunctiva covering the visible scleral surface in the anterior section of the eye. The forniceal conjunctiva connects these two parts. The epithelium contains tight junctions, which are, however, leakier than the corresponding junctions in the corneal epithelium (Ahmed and Patton, 1985). In rabbit, the pore size of the conjunctival

epithelium was shown to be twice that of the corneal epithelium, and the pore density 15 times higher (Hämäläinen et al., 1997). The conjunctival stroma contains the blood and lymphatic flow that can clear the drug to the systemic blood circulation. When penetrating the bulbar conjunctiva, the drug that is not cleared by the blood circulation, reaches the scleral tissue. This non-corneal route is useful mainly for compounds that penetrate the cornea poorly, e.g. very hydrophilic molecules and macromolecules (Ahmed and Patton, 1985; Järvinen et al., 1995). The posterior segment of the eye is also possible to reach through this route, as the drug, after penetrating the sclera, can gain access to the choroidal and retinal tissues. Permeability in the conjunctiva varies around $1 \times 10^{-6} – 5 \times 10^{-5}$ cm/s (Prausnitz and Noonan, 1998; Ramsay et al., 2017), and has been shown to depend on molecular features, such as polar surface area and hydrogen bond donor count (Ramsay et al., 2017). Conjunctival permeability does not, however, directly correlate with the lipophilicity (logD) of the compound (Prausnitz and Noonan, 1998; Ramsay et al., 2017).

Sclera

The sclera consists of the episclera and scleral stroma. In addition, the suprachoroid lamina (lamina fusca), residing inner to the scleral stroma, can either be counted as a part of the sclera or part of the choroid. In reality, it consists of parts of both tissues (Remington, 2012), and forms a loose layer that does not play a role in the barrier functions of the sclera. It can, however, be used as a delivery space for suprachoroidal injections. The episclera is also a loose tissue, but on the outer surface of the sclera. It is vascularized and can participate in clearing drugs to the systemic circulation, but otherwise does not play a role in the barrier properties of the sclera (Chan et al., 2010; Ranta and Urtti, 2006). The scleral stroma is considered to be an avascular tissue; even though some vessels traverse the tissue, it has no capillary system (Remington, 2012). It is formed mainly of collagen fibrils and proteoglycans. The barrier properties and the structure of the sclera are similar to those of the corneal stroma (Prausnitz and Noonan, 1998). Scleral permeability is mainly determined by the molecular size, macromolecules permeating slower than small molecules. Compared to the whole cornea, the sclera is a more permeable tissue (P_{app} for small molecules 5×10^{-6} - 5×10^{-5} cm/s) and the more hydrophilic the drug, the larger seems to be the difference in corneal and scleral permeability (Prausnitz and Noonan, 1998).

Choroid

The choroid is a densely vascularized, loose connective tissue. Its barrier properties in drug permeation are mainly due to its blood flow (Robinson et al., 2006). It has been shown, however, that permeation through the non-

corneal route is not greatly affected by the choroidal blood flow but conjunctival and episcleral blood flow play a more important role (Chan et al., 2010; Robinson et al., 2006). Contrasting reports claim that the choroidal blood flow does play a role (Ranta et al., 2010; Ranta and Urtti, 2006), therefore, more results are needed to assess the relevance of its barrier function in ocular drug distribution, especially in human. The choroid is densely pigmented (Menon et al., 1992), influencing the pharmacokinetics of drugs that bind to melanin pigment. Melanin binding does not contribute to the barrier properties of the tissue as such, but melanin can form a depot of the drug and lower the free concentration in the tissue. It can also prolong the lag time in drug permeation (Pitkänen et al., 2005).

Retinal pigment epithelium

The retinal pigment epithelium (RPE), the outermost layer of the retina, is a densely pigmented cell monolayer with tight junctions (Remington, 2012). It is a part of the blood-retina barrier and has a crucial role in preventing the penetration of xenobiotics to the retina. Permeability in the RPE depends on the lipophilicity of the molecule, more lipophilic molecules permeating faster (Pitkänen et al., 2005). Permeability of small molecules varies approximately from 1×10^{-6} to 1×10^{-5} cm/s, but larger molecules can have permeabilities as low as 3×10^{-8} cm/s (FITC-dextran 80 kDa) (Pitkänen et al., 2005). Compared to the choroid, the RPE is a much greater barrier to drug permeation (Pitkänen et al., 2005; Ranta et al., 2010). Permeabilities of FITC-dextrans were 10-100 fold higher in the excised choroid than in the excised RPE-choroid (unpublished results, presented in Pitkänen et al. 2005). However, the blood flow of the choroid, missing in the excised tissues, increases the barrier function of the choroid. The melanin pigment in the RPE can cause a similar depot effect for binding drugs as mentioned for choroidal pigment, but again, does not participate in the barrier properties.

Vitreous

The vitreous humor, holding a volume of 4 ml in the human eye, is a gel-like material constituting mainly of water (98-99.7%) (Le Goff and Bishop, 2008). In addition, it contains structural collagens, mainly collagen type II, responsible for the gel structure and tensile strength (Le Goff and Bishop, 2008), and glycosaminoglycans, mainly hyaluronic acid, responsible for stabilizing the collagen network (Bu et al., 2015). The mesh size of bovine vitreous has been determined to be around 500 nm (Peeters et al., 2005; Xu et al., 2013) and the vitreous does not pose a barrier for the diffusion of small molecules or even larger macromolecules (therapeutic proteins have diameters less than 10 nm). It can, however, hinder the diffusion of particles larger than 500 nm, such as larger nanoparticles or microparticles for drug

delivery. The vitreous affects the movement of positively charged particles (>500 nm) as the vitreous bears a net negative charge due to the anionic moieties (carboxylic and sulphate groups) of the glycosaminoglycans (Xu et al., 2013). The aging vitreous tends to liquefy, the total fraction of gel decreasing from 80 to ~50% (Bishop, 2000; Sebag, 1987), but this is not expected to significantly modify the barrier properties.

Inner limiting membrane

The inner limiting membrane (ILM), an extrcellular matrix constituted mainly of collagen, laminin and proteoglycans, resides between the retina and vitreous (Halfter et al., 2008; Peynshaert et al., 2017). It is the basement membrane of Müller cells in the retina. It is approximately 3-4 μm thick, except in the foveal area, where it is considerably thinner (min. 20 nm) (Henrich et al., 2012). The ILM can be a barrier for retinal penetration of proteins and nanoparticles (Hutton-Smith et al., 2017; Pitkänen et al., 2004), and the permeation has been shown to depend more on the charge than the size of the permeant (Pitkänen et al., 2004), with positive charge hindering the permeation. For small molecules, the ILM has not been shown to be a barrier.

Blood-ocular barriers

The eye is protected by blood-ocular barriers both in the anterior and posterior segments. The anterior blood-ocular barrier, the blood-aqueous barrier, is composed of the endothelium of the iridial vessels and the non-pigmented epithelium of the ciliary body (Cunha-Vaz, 1979; Cunha-Vaz et al., 2011). These cells have tight junctions that control the entrance of compounds from the blood circulation to the aqueous humor (Figure 1, [3]). The blood-aqueous barrier prevents plasma albumin from entering the aqueous, but small amounts of plasma proteins are present in the aqueous humor due to their passage through the ciliary process capillaries (Schlingemann et al., 1998).

The posterior blood-ocular barrier, the blood-retina barrier, is composed of the RPE and the endothelial cells of the retinal capillaries (Cunha-Vaz et al., 2011). Both cell layers contain tight junctions, efficiently regulating the permeability of ions, water, nutrients, and proteins to the retina. The choroidal capillaries are relatively leaky, allowing passive diffusion of molecules up to 6-12 nm in size (Sarin, 2010). Small molecule drugs and small macromolecules can, therefore, gain access to the choroid, but further penetration through the RPE to the neural retina is limited. The RPE is, nevertheless, permeable to small molecules, especially to lipophilic compounds that can permeate the cell membrane passively (Pitkänen et al., 2005), and drug treatment with small molecules through systemic circulation is possible (Figure 1, [6]). Drug transporters in the RPE can also affect the permeation of this barrier, and consequently affect the distribution of drugs from the systemic circulation to

the retina and vitreous, and vice versa. Although multiple drug transporters, such as multidrug resistance associated protein 1 (MRP1), -4, and -5, have been identified in the RPE (Pelkonen et al., 2017a), their impact on pharmacokinetics is complex and still not fully understood (Vellonen et al., 2017).

2.1.2 DRUG DISTRIBUTION IN THE EYE

The different ocular barriers presented above affect the distribution of drugs in the eye depending on the properties of the drug and the route of drug delivery. Drug distribution between the different ocular tissues is presented in Figure 2.

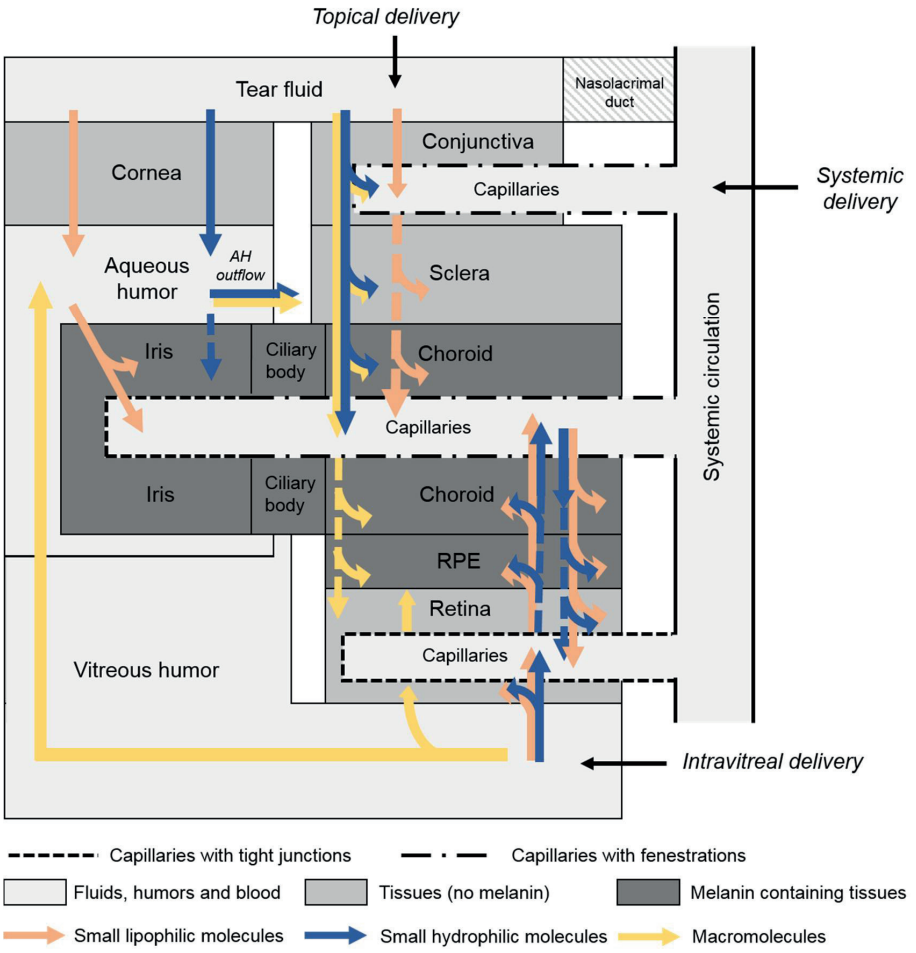

Figure 2 Drug distribution in the eye after topical, systemic and intravitreal delivery. The drug can move between tissues (compartments) that share an edge. The common permeation routes are displayed as arrows (dashed arrow indicates slower permeation). AH, aqueous humor.

Distribution in the anterior segment

Topical drug delivery on the surface of the eye results in effective therapeutic concentrations of drugs only in the anterior tissues; the cornea, conjunctiva, aqueous humor, iris and ciliary body. Drug bioavailability from the ocular surface to the aqueous humor, due to precorneal loss and the corneal barrier function, is generally less than 5% (Maurice and Mishima, 1984). After entering the aqueous humor, the drug is distributed to the inner anterior segment tissues, such as the iris and ciliary body (Figure 2). The volume of distribution (V_d) of drugs in the aqueous humor, measured after an intracameral injection, varies from 2- to 10-fold the volume of the aqueous humor (Durairaj, 2016). Drug exposure to the tissues (cornea, iris, ciliary body) is not necessarily the same as that of the aqueous humor, and the ratio of tissue to aqueous humor exposure has been shown to vary from 1 to at least 800 (Durairaj, 2016). Increased tissue binding, caused by the lipophilicity of the drug or by melanin binding, can increase the levels of drug in the tissues.

Clearance from the aqueous humor is mediated by the blood flow in the iris and the outflow of the aqueous humor (Figure 1, [4], [5]) (Maurice and Mishima, 1984). Hydrophilic compounds are eliminated by the turnover of the aqueous humor (rate of ~3 µl/min, total aqueous volume ~250 µl) (Figure 2). In addition to the outflow, lipophilic compounds are also cleared from the aqueous humor to the surrounding tissues (clearances up to 20-30 µl/min) (Urtti, 2006). The half-lives of drugs in the aqueous humor are relatively short, ranging from 0.4 to 2.2 h in rabbits (Durairaj, 2016).

The drugs that permeate the cornea poorly (hydrophilic molecules and macromolecules), enter the eye through the non-corneal route via the conjunctiva and sclera (Figure 2). After the sclera, the drug faces the choroidal blood flow. Clearance in the choroidal blood flow is dependent on size, small molecules being cleared faster than macromolecules (Kim et al., 2008). It has been shown that the distribution of the drug to the posterior segment tissues after subconjunctival administration is greatly increased by halting the blood circulation by euthanizing the animal or using an *ex vivo* eye (Kim et al., 2004, 2008; Robinson et al., 2006). Thus, reaching therapeutic concentrations for small molecules in the retina is difficult through the sclera, but for larger molecules, such as therapeutic proteins that are cleared by the dynamic flows more slowly, it is an achievable option (Kim et al., 2008; Ranta et al., 2010).

Ocular drug distribution after systemic delivery

The majority of ocular blood flow is in the choroid (Sebag et al., 1994). The choroidal vessels are leaky and small molecule drugs can easily enter the extravascular choroid from the blood flow (Figure 2). Further distribution to the retina, however, is hindered by the RPE (outer blood-retina barrier) (Figure 2). The main factors affecting ocular distribution are unbound drug fraction in the plasma and the distribution clearance from the blood to the

vitreous (Vellonen et al., 2016). The unbound fraction alone does not predict the distribution. The lipophilicity of the drug does not correlate with ocular distribution (Vellonen et al., 2016), even though the permeability in the RPE has been shown to depend on lipophilicity (Pitkänen et al., 2005). The distribution clearance, however, depends on the lipophilicity, together with the hydrogen bonding of the drug, therefore, lipophilicity does play a role in ocular distribution after systemic administration.

The drug can also access the tissues of the anterior segment of the eye through the blood-aqueous barrier. Some ocular diseases, such as glaucoma, can be treated with systemic delivery, but the drawback of this approach is the high systemic concentrations needed to effectively deliver the drug to the eye.

Intravitreal distribution

Treating the diseases of the posterior segment is generally accomplished as intravitreal injections because this can provide drug access to the retina. Vitreous does not pose a barrier for the diffusion of small molecules or even macromolecules and drugs diffuse within the vitreous after injection (del Amo et al., 2017). Vitreal clearance of drugs depends on their capability to pass the blood-ocular barriers; small molecules are cleared mainly via the so called posterior elimination pathways (the blood-ocular barriers) (Figure 1, [8]), and macromolecules, which do not penetrate these barriers easily, are cleared through the anterior route, by the aqueous humor outflow (Figure 1, [9], Figure 2) (Maurice and Mishima, 1984; Urtti, 2006). Lipophilic small molecules, which pass the blood-ocular barriers easily, have the shortest half-lives in the vitreous, generally less than 10 hours (del Amo et al., 2015; Kidron et al., 2012). Hydrophilic small molecules have slightly longer half-lives, up to ~30 h. The half-lives of macromolecules, such as proteins, are much longer than those of small molecules, in the range of several days (Krohne et al., 2012; Zhu et al., 2008). Intravitreal delivery of small molecules as such, without sustained drug delivery systems, is not feasible because of the fast clearance, but protein drugs that have much longer half-lives (e.g. anti-VEGF agents ranibizumab and bevacizumab) can be delivered as such.

The volume of distribution is dependent on the relationship between the total and the free concentration of the drug; binding of the drug in the tissue increases the apparent volume of distribution. The volumes of drug distribution in the rabbit vitreal cavity are comparable to the anatomical volume of the vitreous (del Amo et al., 2015). Therefore, the surrounding tissues do not have a considerable effect on V_d, as their volume is negligible compared to the volume of the vitreous and they are also routes of elimination from the vitreous. The vitreous contains proteins, mainly albumin, and the total protein concentration is estimated to vary between 0.5 and 1.5 mg/ml (Angi et al., 2012; Theocharis et al., 2002; Ulrich et al., 2008). Even though this is much lower than the protein concentration in the plasma (60-80 mg/ml), it is possible that drugs bind to these proteins, affecting the

elimination and level of free drug in the vitreous. Drugs might also bind to other components of the vitreous. The data on vitreal binding is sparse; a few studies have shown unbound fractions from 90 to 99% (Petternel et al., 2004; Schauersberger and Jager, 2002), suggesting that vitreal binding is an insignificant factor in vitreal distribution. More data of this matter, however, is needed to understand its full effect.

2.2 TREATING DISEASES OF THE POSTERIOR EYE SEGMENT

The most serious and prevalent vision-threatening diseases of the eye are the ones of the posterior segment tissues. As mentioned earlier, the aging population is projected to remarkably increase the global prevalence of posterior segment diseases, such as AMD. Treating the posterior segment is thus of increasing interest worldwide.

2.2.1 DISEASES OF THE POSTERIOR SEGMENT

Many of the diseases of the posterior segment of the eye are related to aging, but there are also hereditary dystrophies and ocular infections that exist in the posterior eye.

AMD, the leading cause of blindness in developed countries, is a result of break-down of the macula, the most important area of the retina for visual acuity. It is characterized by the formation of drusen (debris), constituting of various lipids, polysaccharides and glycosaminoglycans, under the RPE (Crabb et al., 2002; Remington, 2012). RPE cells above the drusen are eventually lost and cell death of retinal photoreceptor cells follows overlying the drusen (Ding et al., 2009). AMD can be divided into wet and dry forms. In the wet, neovascular form (wAMD), the capillaries of the choroid grow into the retina through the RPE caused by excessive production of angiogenic growth factors. The dry form does not portray neovascularization, and the formation of drusen is the main characteristic. Of all AMD cases, 10-20% are of the wet form, which progresses much faster than the dry form (Tuuminen et al., 2017). This form can be treated with anti-angiogenic therapies, e.g. antibodies, such as ranibizumab and bevacizumab, or VEGF-trap aflibercept.

Diabetic retinopathy, caused by hyperglycemia leading to degeneration of endothelial cells and thickened retinal capillaries, is a common consequence of diabetes (Cheung et al., 2010). It is characterized by an imbalance in growth factor production that causes angiogenesis. In some cases, the disease can be treated with the above-mentioned anti-angiogenic drugs. Retinopathy is found in up to 80% of patients who have suffered from diabetes for more than 20 years (Stefánsson et al., 2000). The prevalence of DR is much smaller than that of AMD, with an estimated 4.2 million patients globally, but DR is the leading cause of blindness in the working-aged population (20-74 years) in

many industrialized countries, for example in the USA (Cheung et al., 2010). Although the prevalence of diabetes is increasing, the incidence of retinopathy in diabetes patients has been shown to decrease, demonstrating the importance of glycemic control in preventing DR (Klein et al., 2009). Diabetic macular edema (DME), another complication of diabetes, is characterized by inflammation and swelling of the macula due to fluid build-up below the retina (Klaassen et al., 2013). It is less prevalent than DR (Cheung et al., 2010). DME is mainly treated by inflammation reducing intravitreal corticosteroids, but also with the above-mentioned anti-antiogenic therapies.

Retinitis pigmentosa (RP) is a group of heterogeneous genetic retinal disorders that cause death of retinal rod cells responsible for peripheral vision (Dias et al., 2017). It is characterized by the formation of pigmented deposits in the retina. Overall, RP affects about 2.5 million people world-wide. There is no commonly used effective treatment for the disease, but gene therapy seems to be the most promising approach. Voretigene neparvovec (Luxturna), an RPE65 gene therapy, was recently approved for the treatment of RP (FDA, 2017).

Glaucoma can affect the ganglion cell functions in the retina and thereby impair the vision (Schwartz and Budenz, 2004). Glaucoma is usually associated with increased intraocular pressure (IOP) that can cause damage to the optic nerve. Therefore, the symptomatic treatment of glaucoma is based on the reduction of IOP by controlling either the formation or turnover of the aqueous humor. This does not necessarily prevent functional impairment in the retina, and glaucoma is not always associated with elevated IOP (i.e. normotensive glaucoma). Neurotrophic compounds are needed to improve the treatment of glaucoma by maintaining the retinal health of glaucoma patients (Johnson et al., 2011; Nafissi and Foldvari, 2016). The prevalence of glaucoma is slightly less than that of AMD, with an expected 76 million cases globally in 2020 (AMD 200 million) (Tham et al., 2014).

Uveitis, an inflammation of the uvea, can be caused by various disease conditions (e.g. sarcoidosis, hereditary syndromes) as well as infections (e.g. herpes virus, toxoplasmosis) (de Smet et al., 2011). It can be a condition of the anterior or the posterior segment, or both. The causes vary largely, but the treatment aims to reduce inflammation; intravitreal corticosteroids are used for non-infectious uveitis (NIU) in the posterior segment (de Smet et al., 2011).

In addition to the chronic diseases described above, acute infections (endophthalmitis) are a problem of the posterior segment. They may result from surgery, trauma or intravitreal injection, and although rare, they are severe infections that can result in vision loss (Durand, 2017). The causing pathogens vary, and the treatment is planned according to the pathogen. The antibiotics used in treating these infections are usually administered intravitreally (Radhika et al., 2014; Spadea, 2014).

2.2.2 CURRENT STRATEGIES FOR DRUG DELIVERY TO THE POSTERIOR SEGMENT

As the posterior segment tissues are difficult to reach at therapeutic concentrations with topical drug delivery, the current retinal treatments are based on intravitreal injections (del Amo and Urtti, 2008). Delivery through other routes is under investigation and some delivery approaches, such as microinjection to the suprachoroidal space, are already in clinical trials (Pearce et al., 2015).

The first intravitreal drug implant was approved by the United States Food and Drug Administration (FDA) in 1996; a ganciclovir implant for the treatment of cytomegalovirus infection in AIDS patients (Vitrasert, Chiron). It is a polymer-based non-biodegradable implant, designed to release the drug for 5-8 months (Anselmo and Mitragotri, 2014). Other controlled-release products and injectables have since been accepted by the regulatory agencies (Table 1). Small molecular drugs are delivered generally as implants (del Amo and Urtti, 2008); some of the implants can be injected (with a specific device accompanying the implant), and some require surgical implantation (Table 1). Macromolecules, such as anti-VEGF antibodies, due to their low clearance from the vitreous, can be delivered as injectable solutions without a long-acting delivery system. The most problematic are intravitreal small-molecule antibiotics for endophthalmitis, as they are rapidly cleared from the vitreous (Radhika et al., 2014), and for the large variety of antibiotics, there are no commercial long-acting delivery systems on the market. Because of the invasiveness of intravitreal delivery, long-acting delivery systems and sustained treatment strategies are needed for the therapy of posterior segment diseases.

Several sustained release approaches for retinal drug delivery are currently under development and in clinical trials (Pearce et al., 2015). For example, encapsulated cells have been prepared for continuous production of ciliary neurotrophic factor to decrease the loss of photoreceptor cells (Renexus, Neurotech (Birch et al., 2013)). A refillable reservoir, inserted through the sclera into vitreous is being developed for the prolonged delivery of anti-VEGF antibodies (Port Delivery System, ForSight Vision4 (Rubio, 2014)). In addition, a micropump, attached below the episclera, has been developed for prolongation of drug dosing intervals (MicroPump, Replenish (Gutiérrez-Hernández et al., 2014)). These approaches are capable of delivering a variety of drugs in a controlled manner even for years, thereby reducing the number of invasive procedures during the clinical drug treatment. Other methods being investigated for less-invasive delivery to the posterior segment include

Table 1. *Intravitreal treatments for diseases of the posterior eye segment.*

Therapy	Delivery system and administration	Indication	Dosing interval/ Time of effect
Vitrasert (ganciclovir)	Implant, surgical administration	Cytomegalovirus infection in AIDS patients	5-8 months
Retisert (fluocinolone acetonide)	Implant, surgical administration	NIU	30-36 months
Ozurdex (dexamethasone)	Implant, injected	Retinal vein occlusion, NIU, DME	Up to 6 months
Iluvien (fluocinolone acetonide)	Implant, injected	DME	Up to 36 months
Lucentis (ranibizumab)	Solution, injected	wAMD, DR, DME	4-6 weeks
Avastin (bevacizumab)	Solution, injected	Off-label use for wAMD*	4-6 weeks
Eylea (aflibercept)	Solution, injected	wAMD, DR, DME	4-8 weeks
Intravitreal antibiotics[1]	Solution, injected	Endophthalmitis	24-96 h
Amikacin		Aerobic, gram-negative bacteria	24-48 h
Ceftazidime			48-72 h
Vancomycin		Gram-positive, MRSA	72 h

* Originally approved for colorectal cancer. [1] (Radhika et al., 2014)

MRSA, methicillin-resistant *Staphylococcus aureus*

delivery to the suprachoroidal space (lamina fusca) with hollow microneedles (Clearside Biomedical, Inc.) or with a microcatheter (iTrack, iScience Interventional (Tetz et al., 2012)). The active status of research in delivery approaches to the posterior segment of the eye demonstrates the need for developing new strategies to improve the therapy of many vision-threatening diseases. The variety of drug delivery strategies described above is not meant as a comprehensive list, but as a means to shed light on the many developmental approaches to improve the therapy of the posterior eye segment.

2.3 MELANIN PIGMENT

Many drugs accumulate in pigmented tissues, a phenomenon that has been acknowledged for long (Larsson, 1993; Potts, 1964, 1962). The accumulation is a result of drug binding to melanin. Certain ocular tissues are very densely pigmented providing plenty of binding opportunities for melanin binding drugs. Thus, the pigmentation has particularly extensive effects on pharmacokinetics in the eye.

The biological functions of melanin are still not fully understood, although many of its effects have been acknowledged. Melanin absorbs visible and UV light, thus protecting pigmented tissues from the harmful effects of the radiation (Hu et al., 2008). In the eye, the absorption of light improves image quality, as it decreases the reflection of light from the posterior segment tissues. Melanin also acts as an antioxidant by scavenging free radicals and preventing the formation of reactive oxygen species (Bustamante et al., 1993). It binds many endogenous and exogenous chemicals (Ings, 1984; Larsson, 1993; Potts, 1964), participates in metal homeostasis (Hong and Simon, 2007) and thus protects the tissues from the harmful effects of these substances.

2.3.1 MELANIN AND MELANOSOMES

Melanin is a polyanionic polymer located in the pigmented cells, inside intracellular organelles called melanosomes. Melanin can be divided into two types, eumelanin and pheomelanin, based on its molecular structure. Both forms have a common precursor, dopaquinone, oxidized from tyrosine through spontaneous and enzymatic reactions (Figure 3) (Ito and Wakamatsu, 2008; Prota, 1980). In melanogenesis, dopaquinone will be further converted to dihydroxyindole (DHI)-derived subunits that form eumelanin and sulfur-containing benzothiazine-derived subunits for pheomelanin synthesis. Eumelanin is black/brown and pheomelanin of lighter reddish/yellowish color.

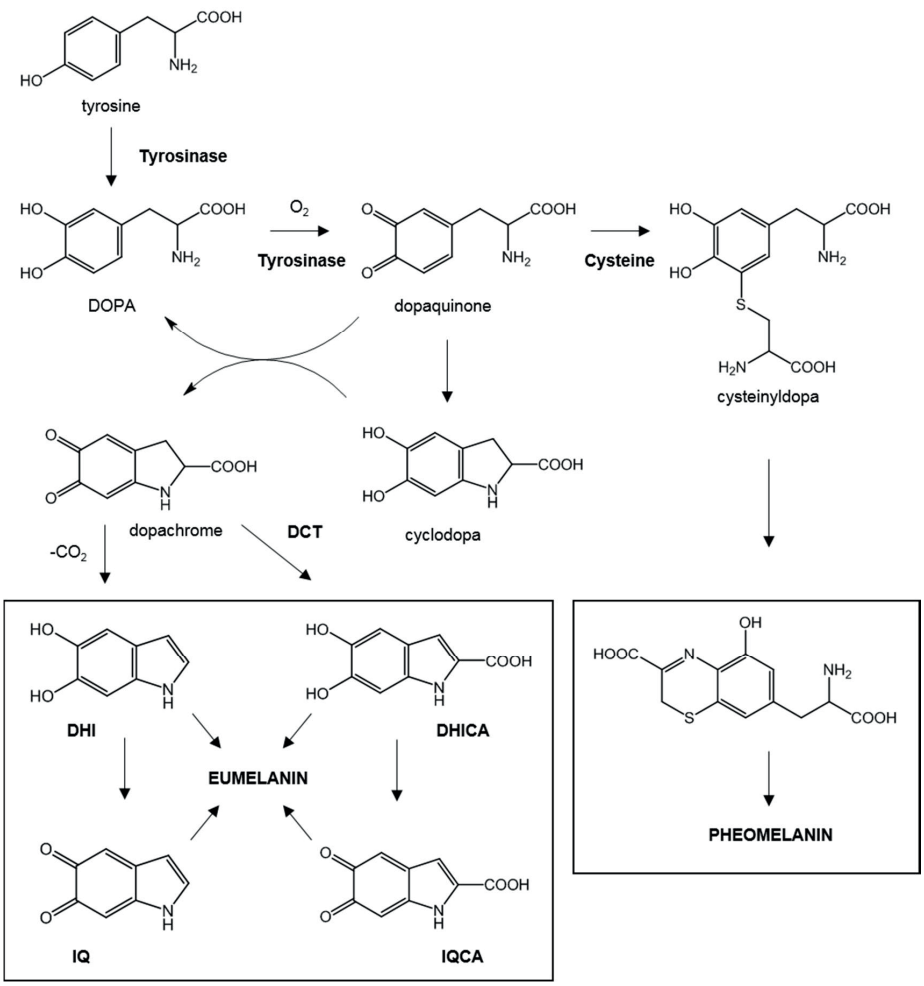

Figure 3 Melanin synthesis and the structure of melanin (modified from (Rimpelä et al., 2017)). Enzymes involved in the synthesis: tyrosinase and dopachrome tautomerase (DCT). DHI, dihydroxyindole; DHICA, dihydroxyindole carboxylic acid; IQ, indolequinone; IQCA, indolequinone carboxylic acid.

Eumelanin is more predominant in ocular tissues (see 2.3.2 Melanin containing tissues) and it is more important for ocular melanin binding of drugs. The structure of eumelanin is heterogeneous and its supramolecular structure is still debated (Meredith and Sarna, 2006). As mentioned above, eumelanin is formed of DHI-derivatives. These subunits can be quinones or semi-quinones and carboxylated or non-carboxylated (Figure 3), and moreover, they are oligomerized through different positions of the subunit molecules, bringing heterogeneity to the molecular structure of eumelanin. Rather than being a linear polymer formed of these subunits, eumelanin is proposed to be formed of oligomers of 4-8 subunits that are stacked as sheets with inter-planar spaces of 3-4 Å (Watt et al., 2009; Zajac et al., 1994). For

drug binding, melanin represents a surface rather than binding pockets. A detailed structure of melanin would benefit the understanding of drug-melanin binding.

Melanosomes are intracellular organelles containing the melanin pigment. Melanin is, therefore, surrounded by a lipid membrane, and it is attached to a protein matrix in the core of the melanosome. Melanosomes bear a resemblance to lysosomes; both share proteins related to, for example, organelle acidification and enzyme digestion, and melanosomes are expected to have a similar acidic pH as lysosomes (Raposo and Marks, 2009).

The lipid composition of melanosomes has been characterized in bovine ocular melanosomes (Ward and Simon, 2007). There were differences in uveal and RPE melanosomes, sphingomyelin being the major melanosomal lipid component in the uvea and glycerophosphoethanolamine in the RPE. Uveal and RPE melanosomes also differ in shape; uveal melanosomes are smaller and round and RPE melanosomes larger and more ellipsoid.

Azarian et al. (2006) characterized the protein content of mature porcine RPE melanosomes identifying 102 proteins related to, for example, melanogenesis, organelle acidification, proteolysis and transporter and channel functions. MRP4 was the only drug transporter found in melanosomes, although some other transport-related proteins were present. More detailed information of melanosome proteome would enable a better understanding of the effect of melanosomal environment in melanin binding and pharmacokinetics.

The pH inside ocular melanosomes is expected to be acidic, but only the pH of non-ocular melanosomes has been characterized (Ancans et al., 2001; Bhatnagar et al., 1993; Fuller et al., 2001). B16 murine melanoma cells have been determined to have melanosomes with intraluminal pH of 3.0-4.6 (Bhatnagar et al., 1993). Skin melanosomes have also been found to be acidic, although a melanosomal neutral pH has been observed in dark-skinned individuals (Ancans et al., 2001; Fuller et al., 2001). Melanin synthesis is proposed to be the most efficient at near neutral pH where the activity of tyrosinase is the highest (Ancans et al., 2001; Ito and Wakamatsu, 2008). Measuring melanosomal pH, however, is difficult, because fluorescence-based methods are hampered by the light absorption by melanin. Knowing the pH of ocular melanosomes would, nevertheless, be important for a deeper understanding of the drug-melanin binding environment.

2.3.2 MELANIN CONTAINING TISSUES

Melanin pigment can be found in various parts of the body; in the eye, brain (substantia nigra and locus coeruleus), inner ear, skin, hair follicles and hair (Dubey & Roulin 2014). Melanin containing cells are generally called melanocytes, and they are dendritic cells. In the eye, melanocytes are present in the choroid and in the stromas of the iris and ciliary body. Ocular pigment epithelial (iris, ciliary body and retinal pigment epithelial) cells are of different

embryonic origin than other pigmented cells, are structurally different (polygonal cells), and are therefore not called melanocytes.

The amount of melanin in different parts of the body varies, but the eye is the most densely pigmented organ (Watts et al. 1981). Melanin content in the tissues can also vary between individuals, as for example skin pigmentation has clear racial differences. Melanin content of ocular tissues varies due to differences in eye color; lighter colored eyes have generally less melanin (35.2 ± 0.9 mg/g of uveal tract in blue eyes vs. 42.8 ± 1.6 mg/g of uveal tract in brown eyes (Menon et al., 1992)). The RPE shows gradually lowering levels of pigmentation in the aging eyes (Feeney-Burns et al., 1984; Schmidt and Peisch, 1986), but racial differences in the amount of melanin are minimal (Weiter et al., 1986). The amount of melanin in the RPE does not vary with eye color (Schmidt and Peisch, 1986), but the total amount of melanin in the posterior eye segment (RPE and choroid) is larger in darker-colored eyes (6.7 mg of melanin/tissue in blue eyes vs. 8.4 mg/tissue in brown eyes) (Menon et al., 1992).

Melanoma tumors constitute a special type of melanin containing tissue. Melanoma is generally caused by excessive UV radiation that causes cancerous changes in the skin melanocytes. These cancerous cells can metastasize throughout the body. Melanoma cells are very densely pigmented, having even higher pigmentation than ocular tissues (Borovanský & Riley 2011). The high-density pigmentation can be used for targeting melanin-binding agents to the tumors (Link et al. 1989).

In the eye, melanin can be found in the stromas of the iris, ciliary body and choroid and in the epithelia of the iris, ciliary body and retina (Figure 4). These tissues form a melanin-containing envelope around the eye. According to Menon et al. (1992) ~60% of ocular melanin is in the RPE-choroid, and both the ciliary body and iris contain ~20% of the total ocular melanin.

The epithelial melanin is mainly eumelanin (Ito et al., 2013; Prota et al., 1998). Melanocytes in the uveal stroma contain both eumelanin and pheomelanin and their ratio seems to correlate with the color of the iris, with more eumelanin in dark-colored eyes (Wakamatsu et al., 2008). The turnover of melanin in ocular tissues is very low; in the RPE, melanin synthesis takes place mainly during embryogenesis and ceases by two years of age (Boulton, 1998). Since melanin synthesis in negligible after birth, the amount of melanin in the RPE decreases with age (Feeney-Burns et al., 1984; Weiter et al., 1986). The melanin content in the RPE of the age group 81-97 was ~40% of that of the age group 14-40.

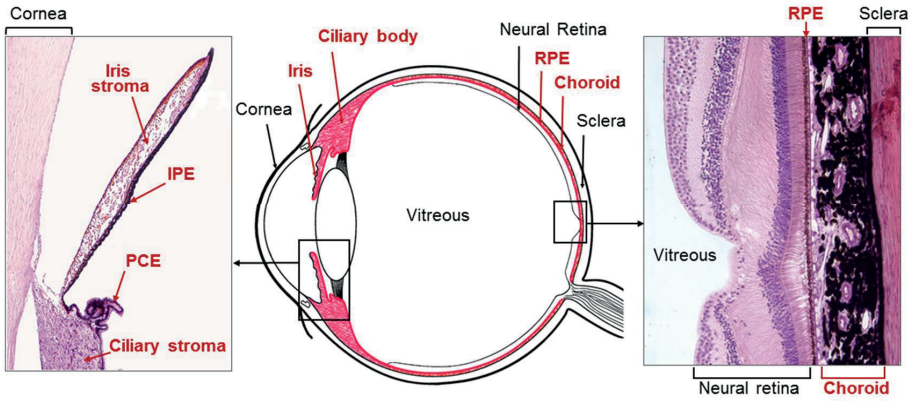

Figure 4 Melanin in ocular tissues. (IPE, iris pigment epithelium; PCE, pigmented ciliary epithelium.) Figure from Rimpelä et al. (2017), reprinted with permission from Elsevier.

2.3.3 MELANIN BINDING OF DRUGS AND ITS IMPLICATIONS IN OCULAR DRUG DELIVERY

Melanin binding of drugs is of interest in drug development, as it is a surprisingly common phenomenon. The first indications of drug-melanin binding arose already in the late 1950s when the accumulation of phenothiazine drugs to the pigmented ocular tissues of animals was discovered (Potts, 1962). These drugs were administered systemically and their ocular accumulation was an unexpected event at the time. Since the recognition of pigment binding as the reason for the accumulation, it has been shown that long retention times, even up to a year after a single systemic dose (shown with chloroquine), are possible (Larsson, 1993; Rosenthal et al., 1978). Since then, a wide selection of compound groups, e.g. beta-blockers, antimalarials, antibiotics, antidepressants, anesthetics and sympathomimetics, have been discovered to contain melanin-binding drugs. Drug development can, therefore, benefit broadly of a deeper understanding of drug-melanin binding.

Although a wide variety of molecular structures can bind to melanin pigment, it has been shown that practically all lipophilic and basic drugs bind to melanin to some extent (Leblanc et al., 1998; Reilly et al., 2015). This is expected, as melanin is a polyanionic and hydrophobic structure. Binding interactions related to drug-melanin binding include hydrophobic interactions and electrostatic interactions, including hydrogen bonding and charge transfer interactions (Kaliszan et al., 1993; Lowrey et al., 1997; Reilly et al., 2015). The molecular features suggested to be relevant for melanin binding are hydrophobicity, aromaticity, charge-transfer properties and relative positive charge (Lowrey et al., 1997; Reilly et al., 2015). The binding interactions of

individual compounds vary, and a general mechanism for binding is unlikely to exist.

The pharmacokinetic and/or pharmacodynamic effects of melanin binding of ocular drugs have been shown in many studies. The most widely studied drugs include topically applied drugs, such as the anti-glaucoma drugs timolol and pilocarpine, and the mydriatic atropine (Araie et al., 1982; Lee and Robinson, 1982; Salazar et al., 1976; Salazar and Patil, 1976; Salminen and Urtti, 1984; Urtti et al., 1984). Ten- to hundred-fold higher concentrations of timolol have been found in pigmented ocular tissues compared to the corresponding albino tissues (Salminen and Urtti, 1984). Similarly, pigmented tissues have contained up to ten-fold higher concentrations of pilocarpine (Lee and Robinson, 1982; Salminen et al., 1984). The IOP-decreasing response to timolol has been shown to differ in pigmented and albino eyes as well as in eyes with different colored irides. Despite the higher total concentrations of the drug in pigmented tissues, the response was lower in pigmented than in albino animals (Nagata et al., 1993) and similarly lower in dark-colored than in light-colored eyes (Salminen et al., 1985). This was presumably due to the reduction in the free drug concentration caused by melanin binding (Salminen et al., 1985). The response to atropine and pilocarpine, however, was prolonged in pigmented animals compared to albino animals, as melanin forms a depot of the drug from which the drug is released slowly (Salazar and Patil, 1976; Urtti et al., 1984). It is clear that melanin binding affects the distribution and pharmacodynamics of binding drugs and that physiological factors, such as tissue melanin content, may affect the drug response. For a more thorough examination of the implications of melanin binding in ocular drug delivery, the reader is referred to the review section of publication III.

Melanin binding has been investigated also as a desired phenomenon. The pigmented tissues of the eye are in close proximity to drug targets of both the anterior and posterior segments of the eye. Targeting melanin could prolong the effect of a drug and also target the drug action to the desired tissues. Melanin binding was shown to increase the retention of orally administered pazopanib, a small molecule anti-VEGF agent, in the uveal tract of pigmented mice compared to albino mice (Robbie et al., 2013). Binding of the drug to melanin was thus able to target the drug to the back of the eye, and prolong the retention of the drug in the posterior segment, showing the feasibility of this approach in sustaining drug action. More studies are, however, needed to evaluate the true potential of this strategy in treating ocular diseases.

All in all, it is crucial to understand that the effects of melanin binding on ocular drug distribution and response are multifaceted, and depend on many pharmacokinetic factors as well as pharmacodynamic properties of the drug; drugs that bind to melanin *in vitro* do not necessarily reach the melanin in the tissues, inside the intracellular melanosomes, and will not bind *in vivo* (Larsson et al., 1981); drugs for ocular and non-ocular indications may accumulate in the ocular tissues (Potts, 1962); melanin binding may protect the tissue from too high concentrations of drugs, but potent drugs may cause

harmful effects because of the accumulation (Leblanc et al., 1998). For these reasons, it is evident that a deeper, systematic understanding of the interplay of different physiological factors, drug properties and melanin binding can benefit the drug development process as well as the understanding of clinical consequences of drugs accumulating to pigmented tissues.

3 AIMS OF THE STUDY

The overall aim of the thesis project was to investigate the effect of melanin and vitreal binding of drugs on their pharmacokinetics in the posterior segment of the eye. This was done using *in vitro*, *in vivo* and *in silico* methods.

The specific aims of the thesis were:

1. To find the most suitable binding isotherm to reliably interpret *in vitro* drug-melanin binding results for calculating maximum binding capacity and affinity (I).

2. To evaluate how well cellular level melanin binding can be predicted from *in vitro* binding studies with isolated melanin (I, II, IV).

3. To study the interplay of cellular factors and melanin binding on the retention of drugs in pigmented tissues of the posterior segment of the eye (II, III, IV).

4. To investigate the effect melanin binding has on intracellular unbound drug concentrations in the RPE (IV).

5. To study drug binding to the vitreous humor and its effect on vitreal pharmacokinetics (V).

4 OVERVIEW OF THE MATERIALS AND METHODS

The materials and methods used in this thesis project are presented in Table 2. More detailed information of the methods can be found in the publications.

Table 2. *Overview of the materials and methods.*

Study (publication)	**Method**
***In vitro* studies**	
Binding of drugs to isolated melanin (I, II, IV)	Melanin isolation from porcine RPE-choroid Zeta potential of melanin (II) Binding assay UPLC and LC-MS/MS Calculation of binding parameters (Wolfram Mathematica and Phoenix WinNonlin)
Studied drugs: Chloroquine, timolol, nadolol, CDCF, methotrexate, propranolol, diclofenac	
Cell uptake and elimination (II, IV)	Isolation and culturing of porcine primary RPE (pRPE) cells ARPE-19 cell culture Uptake and elimination assays Melanin content in the cells by spectrophotometry (IV) UPLC and LC-MS/MS
Studied drugs: *Uptake: Chloroquine, timolol, CDCF (only pRPE), methotrexate, propranolol, diclofenac* *Elimination (only in pRPE): Chloroquine, timolol*	
Binding of drugs to cell homogenate (to determine intracellular unbound drug fraction) (IV)	Isolation of pRPE cells ARPE-19 and pRPE cell culture Binding assay (rapid equilibrium dialysis) Melanin content in the cells by spectrophotometry LC-MS/MS
Studied drugs: Chloroquine, timolol, methotrexate, propranolol, diclofenac	
Binding of drugs to the vitreous (V)	Isolation of porcine vitreous humor Binding assay (rapid equilibrium dialysis) LC-MS/MS
Studied drugs: Cassette mixture of 35 ocular drugs (detailed information in V)	

***In vivo* studies**	
Chloroquine kinetics in the eye (II)	Radiolabeling of chloroquine with ^{123}I SPECT/CT of pigmented and albino rats
***In silico* studies**	
Cell uptake, unbound fraction, and intracellular bioavailability predictions (II, IV)	Calculations in Excel
Kinetic simulations of melanin binding (III)	Stella software
QSAR modeling (III)	KNIME, RDKit, Simca-P software
Kinetic simulations of vitreal binding (V)	Stella software

CDCF, 5(6)-carboxy-2′,7′-dichlorofluorescein; LC-MS/MS, liquid chromatography-tandem mass spectrometry; QSAR, quantitative structure-activity relationship; SPECT/CT; single photon emission computed tomography/computed tomography; UPLC, ultra-performance liquid chromatography

5 PUBLICATION I: INTERPRETATION OF OCULAR MELANIN DRUG BINDING ASSAYS. ALTERNATIVES TO THE MODEL OF MULTIPLE CLASSES OF INDEPENDENT SITES

Reprinted with permission from Manzanares J, Rimpelä A-K, Urtti A: Interpretation of ocular melanin drug binding assays. Alternatives to the model of multiple classes of independent sites. Mol Pharmaceutics 13 (9): 1251-1257, 2016.

6 PUBLICATION II: DRUG DISTRIBUTION TO RETINAL PIGMENT EPITHELIUM: STUDIES ON MELANIN BINDING, CELLULAR KINETICS, AND SINGLE PHOTON EMISSION COMPUTED TOMOGRAPHY/COMPUTED TOMOGRAPHY IMAGING

7 PUBLICATION III: IMPLICATIONS OF MELANIN BINDING IN OCULAR DRUG DELIVERY

Publication III: Appendix A

Can be found online at https://doi.org/10.1016/j.addr.2017.12.008

Publication III: Appendix B

Publication III: Appendix C

8 PUBLICATION IV: MELANIN TARGETING FOR INTRACELLULAR DRUG DELIVERY: QUANTIFICATION OF BOUND AND FREE DRUG IN RETINAL PIGMENT EPITHELIAL CELLS

9 PUBLICATION V: BINDING OF SMALL MOLECULE DRUGS TO PORCINE VITREOUS HUMOR

10 SUMMARY OF THE MAIN RESULTS

The main results of the publications are summarized in Table 3. For the detailed results, the reader is referred to the original publications.

Table 3. *The main results of the thesis.*

***In vitro* melanin binding of drugs (I, II, IV)**	
Suitability of different binding isotherms for analysis of melanin binding results	Melanin binding is relatively non-specific and high capacity binding and should not be represented by the commonly used specific binding isotherms (i.e. Langmuir isotherm for one or two binding site classes). The Sips isotherm was more suitable for calculating maximum binding capacity and affinity, because it takes into account the non-specific nature of binding.
In vitro binding	Basic drugs bound more strongly than acidic drugs (7 drugs studied). pH affected the binding *in vitro*, especially in the case of acidic drugs, which bound more strongly at lower pH (7.4 vs. 5.0).
Studied drugs: Chloroquine, timolol, nadolol, CDCF, methotrexate, propranolol, diclofenac, (I: metoprolol from (Pitkänen et al., 2007)*)*	
Classification model for high and low melanin binders (III)	
QSAR modeling	A classification model that predicts high and low melanin binders was built based on literature binding data. The molecular properties that determined binding were related to polarizability and electrostatic interactions.
The interplay of melanin binding and cellular factors in the retention of drugs in pigmented ocular tissues (III)	
Kinetic simulations of pharmacokinetics in the posterior segment of the eye	The most important factors determining retention in pigmented tissues were the affinity to melanin and cell membrane permeability. Ion trapping also increased the retention. Dissociation rate from melanin played a role with the moderate model binder (timolol) but not with the high model binder (chloroquine). Eye color (differences in the amount of melanin) did not affect drug concentrations in the back of the eye considerably. The unbound concentration of a high-binding drug was only a small fraction (0.1-0.01%) of the total drug concentration in pigmented tissues.
Studied drugs: Chloroquine, timolol (melanin binding parameters in the model)	

Cell uptake and elimination (II, IV)	
Cell uptake	Uptake of the melanin binding drugs was higher in pigmented pRPE cells than non-pigmented ARPE-19 cells, and similar in both cells for the drugs that are low/non-binders. The results for chloroquine varied in the two studies (II, IV), with much lower than expected uptake in IV.
Elimination from pigmented pRPE cells	Chloroquine was retained in the pigmented cells (88% left in the cells after 48 h), but timolol was eliminated faster (more than 85% eliminated within 6 h).
Studied drugs: *Uptake: Chloroquine, timolol, CDCF (only pRPE), methotrexate, propranolol, diclofenac* *Elimination: Chloroquine, timolol*	
Intracellular unbound drug concentrations in RPE cells (IV)	
Intracellular unbound fraction, $f_{u,cell}$	In pigmented pRPE cells, $f_{u,cell}$ varied in the range 0.00016-0.73 and the rank order of the drugs studied could be explained with the drug's affinity to melanin together with lipophilicity. In non-pigmented ARPE-19 cells melanin affinity did not play a role, and $f_{u,cell}$ varied in the range 0.017-1.0.
Intracellular bioavailability, F_{ic}	F_{ic} was similar in both pRPE and ARPE-19 cells, as is expected, since binding should not affect F_{ic}. Previously reported lower values for acidic than basic compounds, were also encountered here.
Studied drugs: Chloroquine, timolol, methotrexate, propranolol, diclofenac	
Predicting cell uptake and intracellular unbound drug concentrations based on *in vitro* binding parameters (II, IV)	
Cell uptake	In general, cell uptake to pRPE cells could be roughly predicted with melanin binding parameters (chloroquine was an outlier in the rank order (IV), due to the unexpectedly low uptake). The correlations were improved when the pH partitioning in acidic subcellular compartments was included in the predictions.
Unbound drug concentration (IV)	The rank order of the unbound drug concentrations of the melanin binding drugs was correctly predicted based on *in vitro* binding parameters. When the low/non-binders were added to the correlation, the rank-order was no longer correctly predicted, due to other cellular binding of the lipophilic diclofenac.
Studied drugs: Chloroquine, timolol, methotrexate, propranolol, diclofenac, CDCF (only uptake, II)	

***In vivo* kinetics of ^{123}I-chloroquine in pigmented and albino rats (II)**	
Ocular retention	^{123}I-chloroquine was retained in the eyes of the pigmented rats but not the albino rat after intravenous administration. SPECT/CT was a suitable imaging method to study melanin-related ocular retention non-invasively.
Binding of drugs to the vitreous (V)	
In vitro binding to the vitreous	Drug binding to porcine vitreous humor was relatively low, in general much lower than melanin binding. Binding varied between 21 and 74% in the fresh vitreous and between 0 and 64% in the frozen vitreous at the concentration 100 ng/ml (35 drugs studied).
Kinetic simulations	Drug binding to the vitreous had only a modest effect on vitreal pharmacokinetics ($AUC_{free\ drug}$ 75-100%, half-lives up to ~3-fold compared to no binding).

11 DISCUSSION AND FUTURE PROSPECTS

Drug delivery to the posterior segment of the eye continues to be a challenge, as the burden of posterior segment diseases is increasing and less invasive or longer-acting drug delivery methods are needed to improve the therapy and decrease the cost of treatment. New strategies to develop drug delivery systems and approaches for sustained action are widely investigated. Using melanin binding as a tool to prolong the action and target the pigmented tissues of the eye could be a feasible approach in ocular drug delivery. This thesis project aimed to study the impact of melanin and vitreal binding of drugs on their pharmacokinetics in the eye by using *in vitro*, *in vivo* and *in silico* tools. In the following sections, the relevance of the methods used in this study as well as the implications of the results and melanin and vitreal binding in general, will be discussed.

11.1 RELIABILITY AND TRANSLATIONAL VALUE OF THE METHODS

11.1.1 MELANIN BINDING METHODS

Melanin binding of drugs can be easily studied *in vitro* with isolated or synthetic melanin. This interaction, however, only portrays the intrinsic interaction with melanin and does not consider other features, such as drug permeability in the melanosomal and plasma membranes and other pharmacokinetic factors. The method is, on the other hand, a simple way to screen and assess the binding early on in the drug discovery and development process. Multiple factors must be taken into account in the binding studies.

First, the pH, at which the binding is studied, can affect the results. The pH values of ocular melanosomes are unknown, and the most relevant pH for binding studies can only be speculated. We studied melanin binding of five compounds to isolated porcine RPE-choroidal melanin at pH values 5.0 and 7.4 (publication II). The binding was pH-dependent, especially for the acidic compounds with pK_a values close to this pH range. The results can be explained based on the properties of melanin and the drugs. Melanin is a negatively charged polymer, with expected pK_a values around 4-5 (Klosterman and Bettinger, 2016), and therefore it will bear less negative charges at the lower pH than at neutral pH. Acidic drugs will undergo a similar shift in ionization being more neutral at the lower pH. Binding interactions are increased at the lower pH environment, because the repulsion of the negative charges of melanin and the drug is decreased. This was observed in our data: melanin binding of methotrexate and CDCF was low at pH 7.4, but increased considerably at pH 5.0. Similar pH-dependent binding has been seen with

acidic compounds before (Tsuchiya et al., 1987). The basic compounds studied did not show pH-dependent binding, as their pK_a values were much higher (>9), and their charge would not change significantly in the pH range 5.0-7.4. Even though the charge density of melanin may change with pH (zeta potential -17.1 ± 1.1 mV at pH 5.0 and -20.3 ± 0.3 mV at pH 7.4), only minimal changes in the binding of the basic compounds were seen.

Secondly, melanin binding is relatively non-specific, low affinity and high capacity binding on a heterogeneous melanin surface. Binding is rarely studied at concentrations high enough to reach saturation and the binding curves are extrapolated to estimate maximum binding capacity (B_{max}) and affinity (K_d) (Koeberle et al., 2003; Pescina et al., 2012; Pitkänen et al., 2007). These parameters have generally been calculated with Langmuir binding isotherms for one or two binding site classes (Koeberle et al., 2003; Ono and Tanaka, 2003; Pescina et al., 2012; Pitkänen et al., 2007), where the binding events are assumed to represent only one or two specific binding energies, portraying binding of a gas to a homogenous surface, or specific binding, for example drug binding to a receptor protein. It is more likely that the binding energies on the heterogeneous melanin surface show a distribution of values, because the binding molecules are large and flexible compared to gas molecules. We considered these aspects and compared the binding parameters calculated with the Langmuir and Sips isotherms (publication I). The Sips isotherm takes into account the distribution of binding energies through a heterogeneity index n. This is in line with the theory of binding on a heterogeneous surface, and we showed that the binding was more reliably analyzed with the Sips isotherm.

Thirdly, the source of melanin can affect the results (Koeberle et al., 2003; Pitkänen et al., 2007). We used only porcine melanin isolated from the RPE-choroid (publications I, II, and IV), but other melanin types, especially synthetic melanin and melanin from *Sepia officinalis* are widely used in binding studies. There are differences in the structure of these melanins, although the structures are not known in detail. Synthetic melanin, produced through oxidation of tyrosine, has been shown to contain mainly uncarboxylated DHI subunits (Ito, 1986), while natural eumelanin contains both DHI and the carboxylated, dihydroxyindole carboxylic acid (DHICA), subunits (Figure 3) (Ito, 1986; Pezzella et al., 1997; Tsukamoto et al., 1992). Particle sizes of different melanins as well as differently isolated melanin from the same source can vary (Liu and Simon, 2003), influencing the surface area available for binding in the *in vitro* study. This, in turn, can affect the binding capacity. Normalizing the binding values to the surface area could help take this into account (Pitkänen et al., 2007), but this would require measuring the surface area in all cases, which can be laborious and require specific instrumentation. Pitkänen et al. (2007) reported 2-4-fold higher binding (nmol/mg of melanin) of betaxolol and metoprolol to synthetic compared to isolated bovine ocular melanin. Normalizing the binding to the surface area of the melanin reduced the difference, but synthetic melanin bound also more

drug per area. They reported higher binding for betaxolol than for metoprolol when the results with the same melanin were compared. Betaxolol binding to bovine melanin was, however, similar to metoprolol binding to synthetic melanin. This was the case also with the surface area normalized results. Therefore, when comparing the results of different drugs, the binding should be studied with the same type of melanin in the same conditions. Nevertheless, more information is needed on the effect of the surface area and melanin structure on the binding, especially for improving the comparison of different *in vitro* studies. We chose to study the binding with porcine RPE-choroidal melanin because our focus of research is in the posterior eye segment, porcine eyes are easily available, and this type of melanin has been previously used in melanin binding studies (Pescina et al., 2012).

The *in silico* methods, QSAR and comparable structure-activity relationship modeling, can be used together with *in vitro* binding to evaluate the relevant molecular features in melanin binding. They can also be used to predict the binding of new compounds, even before they have been synthesized (*in silico* screening of compound libraries). Structure-activity models have been built of *in vitro* (Kaliszan et al., 1993; Radwańska et al., 1995; Reilly et al., 2015) and *in vivo* results (Zane et al. 1990: uveal tract-to-blood ratio of the drug in pigmented rats). The *in vitro* studies have been conducted with the traditional method (incubating the drug with the compound) or modified methods, such as affinity chromatography with melanin immobilized to a high-performance liquid chromatography (HPLC) column and measuring the retention time of the compound. It is important to understand that the model will only describe the outcome that is measured, i.e. retention in a melanin column or traditional *in vitro* binding. It has the same limitations of *in vitro* studies as explained above, and only describes the intrinsic interaction under certain conditions. When *in vivo* data is used for the analysis, the outcome the model predicts is closer to the situation in the body, but the phenomenon predicted here is much more multifaceted and can add uncertainty to the model. Another concern of the QSAR approach is related to the chemical space of the used compound set (Eriksson et al., 2003). The published models have had only narrow chemical spaces of molecular analogues, and these types of models are not applicable outside their chemical space. Because melanin binding is relatively non-specific, a large data set is required to build a generalized model. The authors of Pelkonen et al. (2017b) were not able to build a QSAR model based on the *in vitro* melanin binding results of 34 drug compounds studied as a cassette; the compound set had a relatively wide chemical space, possibly affecting the failure to build a quantitative model of a small drug set. Jakubiak et al. (2017) were able to build a categorizing model with *in vitro* binding results of 3500 compounds with a 90 % success rate in categorizing new compounds (model not published, reference from a conference). This shows that large compound sets are needed to make predictive models of melanin binding. The model built by Reilly et al. (Reilly et al., 2015) included the data of 263 compounds, but the model

equation was not published and, therefore, cannot be used publicly. We aimed to build a classification model that can distinguish between high and low melanin binders (*in vitro*), with the purpose of helping to assess the likelihood of a certain compound to bind to melanin (publication III). Classification instead of a purely quantitative approach, simplifies the model, giving an easy-to-interpret tool for evaluating drug binding to melanin for *in silico* screening. Building a quantitative model based on literature data would be impossible, due to the wide variety of experimental conditions used.

Experiments with pigmented cells also take into account the other processes involved in melanin binding, such as permeability in the membranes and melanosomal pH. This approach has been utilized only rarely, and data from cell uptake studies are scarce. Many continuous cell models, for example the RPE cell line ARPE-19, are generally non-pigmented at short culture times (<2 months) (Ahmado et al., 2011). Therefore, the use of primary cells containing melanin pigment is justified. We studied drug uptake and elimination in porcine primary RPE cells (publications II and IV). Porcine eyes are convenient to use for their easy availability from slaughter houses and the simplicity of RPE cell isolation. RPE cells, however, do not synthesize melanin in the normal conditions, and, therefore, the amount of melanin in the *ex vivo* cells is distributed between the daughter cells upon cell division. Thus, the final melanin content will be smaller than in the intact RPE *in vivo*. Measuring the amount of melanin in the cells is important when the effect of melanin on cellular drug uptake or elimination is studied, for a better experimental comparison between different cell batches and cell types. Quantitating the amount of melanin can also give insight into the effect the amount of melanin has on drug uptake. In addition to primary cells from animal eyes, for example human fetal RPE cells and ARPE-19 cells fed with melanin or melanosomes can be used (Basu et al., 1989). Melanin and melanosomes are phagocytosed by ARPE-19 cells and the amount of melanin inside the cells can be controlled (Rimpelä et al., 2017). These cells were not utilized in the experiments of this thesis project, but they are promising tools to evaluate pigment related drug pharmacokinetics at the cellular level.

We employed a method that has been previously used with non-pigmented cells (Mateus et al., 2017b, 2014, 2013), to study the fraction of unbound drug in pigmented cells (publication IV). The unbound fraction (and concentration) of the drug can be considered as the fraction that elicits the drug actions, either beneficial or adverse. From the pigment targeting and sustained action point of view, the unbound concentration determines the feasibility of the approach in a drug-specific manner considering the potency (the concentration that is required for the effect) of the drug. Therefore, it is important to determine the unbound fractions of the melanin-binding drugs in the cells. The method used here entails the incubation of the drug with homogenized cells and measuring both the free and bound drug. Although the method gives useful information of the expected unbound fractions, it does not take into account certain physiological factors, such as intra-melanosomal pH. As a benefit, the method

informs also of other, non-melanosomal binding in the cells. Therefore, the method is a valuable addition to the arsenal of techniques to evaluate the effects of pigment binding.

Although *in vitro* binding studies and cell studies can be useful tools in screening drugs and understanding the intrinsic and cellular level binding, *in vivo* studies are required to take into account the full extent of factors affecting the binding. We demonstrated the use of SPECT/CT imaging in evaluating melanin binding related accumulation of intravenously administered ^{123}I-chloroquine in pigmented and albino rats (publication II). This method is noninvasive, as the same animal can be examined throughout the study. Studying pigment binding *in vivo* is generally accomplished by euthanizing the animal at a certain time point and measuring the concentration in the tissues by other analysis methods, such as LC-MS, HPLC (Cheruvu et al., 2008; Sauer and Anderson, 1994), or autoradiography (Báthory et al., 1987; Lindquist, 1973). These approaches require the use of numerous animals. Non-invasive imaging methods, in contrast, can significantly reduce the number of animals needed. Although tissue-specific concentrations cannot be obtained with the sole SPECT/CT imaging (the resolution does not separate different tissue segments), drug accumulation to the ocular area can be clearly monitored. The kinetics in the ocular area can therefore be assessed. A drawback of this method is the requirement for radiolabeling, a modification that may potentially affect the melanin binding and other properties of the drug.

In addition to *in vitro* and *in vivo* methods, we utilized modeling to evaluate the interplay of melanin binding and cellular and physiological factors in the pharmacokinetics of the posterior eye segment (publication III). With this method, we were able to evaluate the effects of factors such as permeability in the plasma and melanosomal membranes, amount of cellular melanin and melanin binding affinity of the drug, on the retention of drugs in pigmented tissues. This method helps to demonstrate the integrated effects of multiple factors simultaneously and to assess the impact of individual parameters. So far these mechanistic bottom-up simulations have not been experimentally validated in *in vivo* studies. *In vivo* data would help to improve the model for a wider use in assessing the kinetics of binding.

11.1.2 VITREAL BINDING METHODS

Binding of drugs to the vitreous humor was studied with fresh and frozen homogenized vitreous from porcine eyes (publication V). The homogenization process can affect the rheology and composition of the vitreous, as mainly the soluble components are included in the final fraction. Soluble proteins, such as albumin and α_1-acid glycoprotein as well as soluble glycosaminoglycans, components that the drugs could bind to, are expected to be in the final isolate. Therefore, the method can be assumed to be suitable for studying the binding. The data on vitreal binding is scarce (Petternel et al., 2004; Schauersberger

and Jager, 2002), but based on the low volumes of distribution in the vitreous reported in literature (del Amo and Urtti, 2015; Kidron et al., 2012) indicating a low effect of vitreal binding, we can assume the binding analyzed with this method to be close to reality.

11.2 EFFECT OF BINDING ON PHARMACOKINETICS

11.2.1 INSIGHTS FROM THIS STUDY

This thesis project aimed to provide information and increase the understanding of the effect of melanin and vitreal binding on the pharmacokinetics of drugs in the eye. We demonstrated the usefulness of different methods to characterize drug binding to melanin and the vitreous humor. The results give insights into the pharmacokinetic impact of the binding events.

In vitro binding to isolated or synthetic melanin is generally accepted as the measure of melanin binding, as it should be, but with certain reservations. As discussed earlier, the experimental conditions and the source of melanin may affect the results and the simple binding experiment does not take into account other physiological processes. We aimed to bridge the gap between *in vitro* and cellular level melanin binding by comparing the measured cell uptake and unbound fractions in pigmented primary RPE cells with values calculated based on *in vitro* binding parameters, B_{max}, K_d, and n (publications II and IV). Melanin binding, as expected, did not explain the whole uptake, as other cellular factors contribute to it, but the rank order of the amount of drug taken up by the cells was correctly predicted. The correlation was improved, when the pH partitioning in different intracellular compartments calculated based on the pK_a values of the compounds, was included. Additional factors that should improve the correlation include binding to other cellular components, governed mainly by compound lipophilicity (Mateus et al., 2013).

The pharmacokinetic modeling to assess drug retention in the pigmented tissues also takes into account other cellular and physiological factors that act in concert with melanin binding (publication III). The most important factors include melanin binding affinity and capacity (B_{max}, K_d, n): the simulations with binding parameters of chloroquine (high binder) showed much longer retention than the simulations with the parameters of timolol (moderate binder). Cell and melanosome membrane permeabilities also affected the retention considerably; the lower the permeability, the longer the duration of the retention. Ion trapping is described by the preferential distribution and retention of positively charged drugs in the acidic intra-cellular organelles (e.g. lysosomes). This mechanism may be involved in the melanosomal drug entrapment: simulations with lower outward than inward permeability in the melanosome membrane resulted in increased drug retention in the melanosomes. The pH of melanosomes is not known, but can be expected to

be acidic (Ancans et al., 2001; Bhatnagar et al., 1993; Ito and Wakamatsu, 2008). Therefore, ion trapping may increase the retention of basic drugs in pigmented cells.

Eye color is related to the differences in the amount and type of melanin (eumelanin/pheomelanin) in the iris (Eagle, 1988; Wakamatsu et al., 2008). Based on the simulations (publication III), the amount of melanin in different-colored eyes had minimal effect on drug retention in the retina-choroid, since the differences in the amount of melanin in these tissues, are only modest (6.7 mg in blue eyes vs. 8.4 mg in brown eyes). However, the data on melanin amounts are rather sparse, and definite conclusions of the amount cannot be made, especially in the case of disease and aging. In the anterior segment of the eye, *in vivo* studies in human have shown differences in the response to timolol (Salminen et al., 1985), although the difference in the amount of melanin in the anterior tissues, iris and ciliary body, seems to be similar as in the posterior segment (4.2 mg in blue eyes vs. 4.9 mg in brown eyes (Menon et al., 1992)). Therefore, more data of the effect of the melanin amount in the tissues are needed.

The pharmacokinetic modeling demonstrated another crucial consequence of binding; the free concentration is much lower than the total concentration in the pigmented tissue. The cellular free concentration of the high binder (chloroquine parameters) was 0.01-0.1% of the total concentration and of the moderate binder (timolol parameters) 1-10% of the total concentration. Therefore, the total concentration is not representative of the pharmacologically relevant drug retention in the tissue. In pharmacokinetic *in vivo* studies, the total concentration has generally been measured (Lindquist, 1973; Ono et al., 2003; Salminen et al., 1984), and it is important to understand that this differs significantly from the active unbound concentration.

Although tissue binding and unbound fractions in non-pigmented cells have been investigated (Mateus et al., 2017a, 2013; Pfeifer et al., 2013), the unbound fractions and drug binding in pigmented cells have not been studied previously. We studied the unbound fractions in pigmented (primary porcine) and non-pigmented RPE (ARPE-19) cells, demonstrating clear differences due to melanin binding of the drugs (publication IV). In pigmented RPE cells, the unbound fraction of chloroquine, the highest melanin binder studied in this thesis, was only 0.02-0.05% of the total concentration, while in non-pigmented ARPE-19 cells it was 5-10% of the total. This clearly shows the effect pigment binding has on the unbound concentration, especially in the case of extensive binding, and demonstrates the importance of the unbound concentration in cellular and *in vivo* studies. With this method, we could also show, that other cellular binding events, not only melanin binding, decrease the unbound fraction.

The retention of ^{123}I-chloroquine in ocular tissues of pigmented but not albino rats was demonstrated with SPECT/CT imaging (publication II). However, the total concentration was measured, which, again, does not

represent the effective unbound concentration. The retention of total chloroquine (free + bound) in pigmented tissues was shown also in the cell uptake and elimination study. Chloroquine exhibits similar retinal toxicity in both pigmented and albino animals (Leblanc et al., 1998). This suggests that the total accumulation of chloroquine caused by melanin binding does not increase the toxicity of chloroquine, likely because the unbound concentration is still relatively low although the duration of retention is longer.

Based on the relatively low vitreal binding of the drugs evaluated in this study (publication V), it is likely that vitreal binding does not considerably affect the pharmacokinetics or pharmacodynamics of small molecule drugs in ocular tissues. Vitreal binding was much lower than melanin binding; vitreal free fractions being 26% or higher, compared to pigmented cell free fractions being as low as 0.02%. According to the kinetic simulations performed based on the measured vitreal binding, the elimination half-life was increased ~3-fold in the case of the highest binding (74%) compared to no binding. In this case, the area under the concentration curve (AUC, of the free concentration) was decreased to 75% compared to the no-binding scenario, i.e. only a modest change in drug exposure. Data on vitreal binding, however, are scarce (Petternel et al., 2004; Schauersberger and Jager, 2002) and more results could help confirm our finding of the low impact of binding on vitreal pharmacokinetics. Vitreal binding has, for example, been demonstrated as a suitable targeting approach to sustain the action of anti-VEGF agents through hyaluronan-binding peptide moieties (Ghosh et al., 2017), but small molecule drugs have not been shown to bind to the vitreous extensively. We can conclude that the broad pharmacokinetic and pharmacodynamic effects caused by melanin binding do not apply to vitreal binding, and it can be construed as a minor factor in ocular pharmacokinetics.

All in all, we can summarize the main insights from our results to be 1) melanin binding explains cellular retention, as higher binding drugs are retained to a higher extent and can be retained for long, 2) other drug properties (lipophilicity, acid/base status and pK_a, membrane permeability) are important features to consider and explain drug distribution to the cells, 3) free concentrations of melanin-binding drugs are much lower than the total tissue concentration, and emphasis on the free concentration should be given accordingly, and 4) vitreal binding plays a modest role in ocular pharmacokinetics.

11.2.2 GENERAL CONSIDERATIONS

As emphasized in the previous chapters, the effect of melanin binding on the pharmacokinetics is multifaceted. Systemically administered drugs distribute widely in the body, and may accumulate to many tissues that contain melanin, depending on the binding properties and membrane permeability of the drug. The physicochemical properties of the drug determine permeability, and the drug's ability to access melanin within the intracellular melanosomes. The

processes involved in posterior eye segment pharmacokinetics, as related to drug binding to melanin and the vitreous, are compiled in Figure 5.

Distribution of the drug from the systemic circulation to the tissues is governed by plasma protein binding, membrane permeability and tissue binding (Rowland and Towzer, 2011). It has been shown that the unbound fraction of the drug distributes from the plasma to the eye; the lower the plasma protein binding, the higher is the distribution (Vellonen et al., 2016). In addition to ocular distribution, the drug can access other melanin containing tissues and bind to melanin. The eye, however, is the most densely pigmented organ in the body, and the accumulation to the ocular pigmented tissues is expected to be the most extensive. Distribution in other melanin containing tissues has been detected; more extensive drug distribution to the pigmented rat skin compared to non-pigmented skin was seen with basic melanin-binding drugs (comparison of the distribution of 200 structurally differing compounds) but the distribution of these drugs to pigmented ocular tissues was still more extensive (Harrell et al., 2015). The higher distribution to the eye than other melanin-containing tissues has been observed also in other studies (Ono et al., 2003; Tanaka et al., 2004). Melanin binding has, nevertheless, been connected with side-effects in non-ocular melanin-containing tissues: melanin binding of aminoglycoside antibiotics has been suggested to be the reason for their ototoxicity (Wrześniok et al., 2013), dermal side effects of anthracycline therapy have been considered to depend on melanin binding (Svensson et al., 2003), and neurodegenerative properties of the pesticide paraquat and its analogue MPTP have been speculated to be caused by neuromelanin binding in the substantia nigra of the brain (Karlsson and Lindquist, 2016). More information is, therefore, needed on the distribution of drugs to non-ocular pigmented tissues and the effects they may have in these tissues. Drug distribution to the other melanin-containing tissues can be decreased by using local routes of drug administration. Intravitreal delivery, especially, results in higher drug concentrations in the posterior segment of the eye.

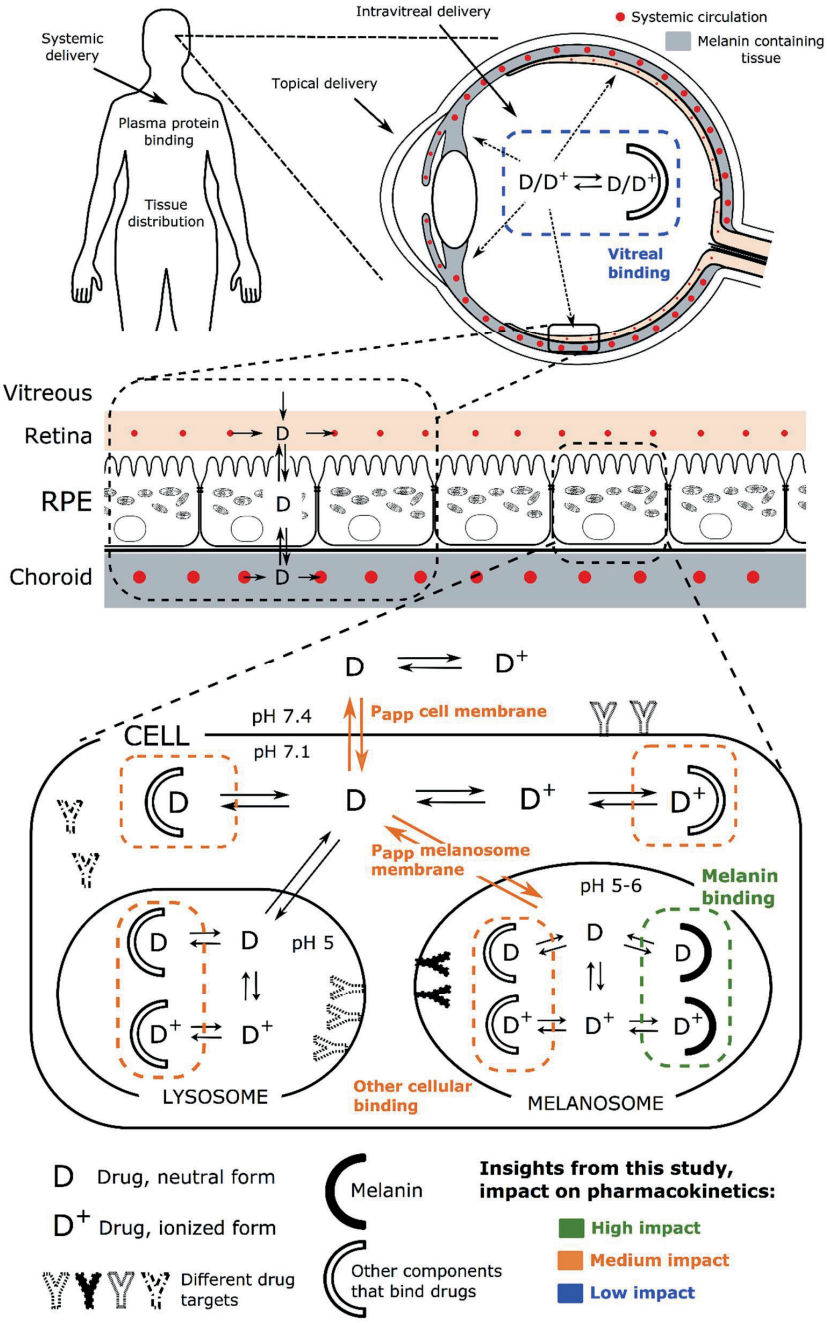

Figure 5 Insights on ocular pharmacokinetics: impact of factors affecting drug distribution and retention in pigmented tissues. Melanin binding was the main contributor to ocular and intracellular distribution of the factors studied (i.e. high impact). Cell membrane permeability and binding to other cellular components than melanin affected ocular and intracellular distribution, but to a lesser extent than melanin binding (i.e. medium impact). Vitreal binding was relatively low and is expected to have only a modest impact on ocular pharmacokinetics (i.e. low impact).

After the drug has reached the cells, intracellular distribution influences the response to the drug (Figure 5). Target location inside the cells and drug partitioning into different compartments of the cell, such as the melanosomes, lysosomes and other endosomes, determines the response. Lysosomes may, for example, accumulate drugs due to the entrapment of positively charged compounds in their acidic lumen (Nadanaciva et al., 2011). This can cause harmful effects, but diseases, such as AMD, that have possible lysosomal targets (Kinnunen et al., 2012), may also benefit from the accumulation. Uncertainty in target exposure is one of the main contributors to failure in clinical trials (Cook et al., 2014; Morgan et al., 2012). As many as 100% of the development programs of compounds where target exposure has not been verified, have been shown to fail in phase II (efficacy study) of the clinical trials (Morgan et al., 2012). Therefore, it is crucial to understand and investigate intracellular distribution of drug candidates. The multifactorial mechanisms involved in drug distribution and melanin binding (Figure 5) demonstrate that mechanistic understanding of intracellular as well as body-level pharmacokinetics is of high importance when evaluating the distribution and effects in pigmented cells.

The drug response in the pigmented tissues depends on the melanin binding properties as well as on the pharmacodynamic properties of the drug. The free concentrations of melanin binding drugs in pigmented tissues can be very similar to those in non-pigmented tissues, even though the total concentration in pigmented tissues is higher. Therefore, the response to the drug may not be affected even though the distribution is. In general (as seen in the kinetic simulations of this study, publication III), melanin binding lowers the maximal cellular free concentration, but the concentrations are retained longer. Toxicity related to melanin binding depends on the same issues described above, i.e. whether the free concentration is sufficient to cause toxicity and if the duration of the retention affects the toxicity. It has been clearly shown that melanin binding in ocular tissues does not predict ocular toxicity (Leblanc et al., 1998), therefore, the toxicological implications need to be assessed individually for each drug.

There is a possibility of drug-drug interactions related to melanin binding as various compounds have been shown to be displaced by other binding compounds in *in vitro* studies (Pelkonen et al., 2017b; Salazar-Bookaman et al., 1994; Testorf et al., 2001). Competitive binding has also been shown to increase the cytotoxic effects of anthracyclines *in vitro*; chloroquine displaced the melanin-bound anthracyclines which were able to consequently cause higher cytotoxicity (Svensson et al., 2003). From the targeting point of view, being displaced by another drug could hamper the effectiveness of targeting as the victim drug would be removed from the desired location. Displacement could, however, also increase the effect as more of the drug would be in the free form. Drug-drug interactions are, therefore, complex, and can be beneficial or counterproductive, depending on whether melanin binding

improves or inhibits the effect and how the other binding drug modulates the free concentration of the victim drug.

This thesis project studied the impact of melanin binding of small molecule drugs, but many of the posterior segment diseases (e.g. AMD and DR) are treated with therapeutic proteins. Macromolecule binding to melanin pigment has been shown with cytochrome c (12.4 kDa), which bound to melanin *in vitro* and had a permeation lag time related to melanin binding in *ex vivo* sclera-choroid-Bruch's membrane (Tratta et al., 2014). Therefore, it is possible that also macromolecules are affected by melanin binding related pharmacokinetic effects, but due to the poor cell membrane permeation of macromolecules, the restriction of access to melanin will most likely hinder the binding *in vivo*. More studies are, however, needed to better assess the impact of melanin binding of macromolecules.

The approach of targeting melanin pigment to achieve sustained action in the anterior and posterior segments of the eye seems to be a promising approach, and indications of its feasibility in the posterior eye segment have already been reported (Robbie et al., 2013). As mentioned above, the eye is the most densely pigmented organ, providing a basis of delivering drugs systemically to reach the pigmented ocular tissues and avoiding harmful accumulation into other pigmented tissues. Melanoma tumors have been targeted with the approach of delivering melanin-binding radiochemicals and other cytotoxic agents to the tumor but avoiding their harmful effects in other pigmented tissues (Link et al., 1989). Melanoma tissue, depending on the type of melanoma, can be more densely pigmented than the eye (mouse melanoma 0.68 ± 0.13% melanin by weight vs. mouse eye 0.40 ± 0.02% (Watts et al., 1981)), and a similar principle is applied there as would be in ocular targeting through systemic delivery; the drug can be targeted to the most densely pigmented tissue and too high concentrations in the less pigmented tissues can be avoided. Another important notion benefiting the approach of targeting through systemic delivery, is that melanin binding does not correlate with plasma protein binding (Pelkonen et al., 2017b). This can ensure a large enough distribution of melanin-binding drugs to ocular tissues from the systemic circulation, as the plasma protein binding is not increased in the same proportion with melanin binding.

11.3 RELEVANCE FOR DRUG DEVELOPMENT

Drug discovery and development processes are daunting tasks lasting for more than a decade in average for each new drug molecule. The cost has been estimated to be even $5 billion per drug (Herper, 2012). Typically about 90% of the compounds that reach clinical phases fail before market authorization.

More efficient preclinical drug discovery and development methods are constantly investigated to improve the success rate in the clinical phases and overall drug development. Recently, a lot of attention has been paid to

computational methods to alleviate the costs of experimental studies and to predict the efficacy and safety of the compounds in man (Jones et al., 2015; Sliwoski et al., 2014). Moving towards computational methods is also in accordance with the principle of the three R's; reduction, refinement, and replacement of animal studies in research. These methods include pharmacokinetic and pharmacodynamic modeling as well as quantitative and mechanistic modeling of interactions of drug molecules with different components (receptors, other proteins, cell membranes, DNA etc.) of the body. This study delivers new computational methods on melanin binding and its pharmacokinetic implications, for the use in drug discovery and development.

For the development of drugs with non-ocular targets and indications, accumulation to the pigmented ocular tissues is considered to be a burden. The possibility of toxicity related to retinal drug accumulation may be considered as too high a risk to take and, therefore, the analogues without accumulation are preferred in drug development. Since new drug candidates tend to be more lipophilic than before (Leeson and Springthorpe, 2007), a feature that also promotes melanin binding, there is a prospect for increased melanin binding of drug candidates in the future. The QSAR classification model built in this study can be used to evaluate melanin binding already at the drug discovery phase to assess if the molecule is classified as a high binder. The molecules shown as high binders, can in turn be tested with *in vitro* methods (*in vitro* binding, cell uptake) to assess the extent of binding.

With binding parameters from *in vitro* studies, the pharmacokinetic model built in this thesis project offers another tool to predict the retention of melanin binding drug candidates in the pigmented tissues. The model can incorporate measured or predicted parameters regarding melanin binding, cellular permeability, ionization inside acidic compartments (ion trapping) as well as macroscopic pharmacokinetic parameters (clearance, volume of distribution) in the vitreous and in the systemic compartment. Therefore, the model reported here can easily be used in early screening in the drug development process.

For the realization of the approach of targeting melanin to obtain sustained action in the pigmented ocular tissues, the unbound fraction of the drug plays a crucial role. Assessing the unbound fractions in pigmented cells in a high-throughput manner during the drug development process would enable the screening of compounds in regard to whether the unbound concentration is sufficient to elicit the drug response. The cell homogenate binding method employed in this study has already been used with a screening capacity of 1000 compounds per week with non-pigmented cells from different sources (Mateus et al., 2017a). Using pigmented cells would require a continuous cell source where pigmentation is at the level of *in vivo* pigmentation in a reproducible manner. Isolated primary RPE cells do not fill these criteria, therefore, different cell sources, such as melanin-fed ARPE-19 cells (Basu et al., 1989; Rimpelä et al., 2017) or stem cell derived RPE cells (Sorkio et al.,

2014), should be explored for this purpose. Nevertheless, this method offers a possibility for the rapid evaluation of the unbound fractions.

11.4 FUTURE PROSPECTS

This study provides new information on the effects of cellular processes in melanin binding, but a more systematic understanding of drug-melanin binding interactions and interplay of cellular and physiological factors would enable a more thorough conception of the factors driving the binding.

The pH of ocular melanosomes still remains unknown and the environment where the binding takes place is not fully characterized. To improve the understanding acquired from *in vitro* studies with melanin and to optimize the binding conditions to correlate with the *in vivo* situation, more detailed information about the pH and the composition of melanosomes as well as the surface area of melanin is needed. Studying the interactions with melanosomes instead of melanin is another option, but the isolation of intact melanosomes is more laborious (Pelkonen et al., 2016) than the isolation of melanin or RPE cells. Thus, studying the binding in whole cells would better assess the interactions in the physiological environment. Furthermore, cell models with a continuous pigmentation comparable to *in vivo* pigmentation would simplify the methods of studying cellular binding, uptake and elimination.

According to the principle of the three R's, and in search of more efficient drug development methods, creating a well-validated ocular pharmacokinetic model would be of great benefit. As melanin binding is an important factor in ocular pharmacokinetics, it should be included in the model with an accurate representation. The pharmacokinetic model could eventually be widened to a PK/PD model, portraying the pharmacodynamic (PD) effect together with the pharmacokinetics (PK). This kind of a model could help in assessing beneficial effects of melanin binding as well as toxicological implications.

Regarding the melanin targeting approach, there are still many hurdles to overcome. In addition to the more systematic understanding of melanin binding and the interplay of other factors with melanin binding, the feasibility of this approach regarding its safety and efficacy aspects still needs to be demonstrated. When this is accomplished, the approach could be used in the treatment of a multitude of diseases with new melanin binding drugs as well as melanin targeting fragments conjugated to existing effective drugs.

12 CONCLUSIONS

This thesis focused on investigating the impact of melanin and vitreal binding of drugs on their pharmacokinetics in the eye. The following conclusions can be drawn from the results of this study:

1) Basic drugs have higher *in vitro* binding to melanin than acidic drugs, due to electrostatic interactions. The *in vitro* binding environment (pH, concentrations of melanin and the drug), however, has a clear effect on drug-melanin binding.

2) The Sips binding isotherm is more suitable for the analysis of melanin binding parameters than the generally used Langmuir binding isotherms, because it takes into account the distribution of binding energies likely to be encountered in melanin binding.

3) Intracellular unbound concentrations of melanin-binding drugs are much lower than the total intracellular concentration, and the total concentration does not necessarily translate into drug response.

4) *In vitro* melanin binding parameters can explain cellular uptake and unbound intracellular fractions of drugs, together with other parameters, such as drug lipophilicity and acid/base status.

5) The interplay of cellular factors with melanin binding is crucial in ocular pharmacokinetics. Cell and melanosome membrane permeabilities and ion trapping into acidic subcellular compartments, in addition to melanin affinity, affected the retention of drugs in pigmented tissues of the posterior segment of eye (RPE-choroid), based on kinetic modeling.

6) Melanin binding retains drugs in pigmented tissues. The high melanin-binding drug, chloroquine, was retained in ocular tissues of a pigmented rat, but not albino rat. Similar retention was seen in pigmented RPE cells *in vitro*.

7) SPECT/CT imaging is a suitable method to monitor melanin binding related ocular drug distribution and retention in pigmented rats.

8) Vitreal binding of small molecule drugs is a less significant factor in ocular pharmacokinetics than melanin binding, as vitreal binding of 35 clinical drugs was found to be relatively low.

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