OCULAR MELANIN BINDING OF DRUGS: *IN VITRO* BINDING STUDIES COMBINED TO A PHARMACOKINETIC MODEL

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Tiivistelmä/Referat – Abstract

Certain drugs accumulate into pigmented tissues due to their binding to melanin, a macromolecule inside pigmented cells. Melanin can affect the drug's pharmacokinetics by acting as a drug reservoir. Binding can also cause toxic effects by accumulating compounds to pigmented cells. This thesis focuses on ocular melanin. The literature review covers the most common methods used in the study of ocular melanin binding and concentrates on *in vitro* methods and the analysis and usability of the results in pharmacokinetic modeling.

The aim of the experimental part was to study melanin binding of a set of compounds *in vitro* with melanin isolated from the retinal pigment epithelium (RPE) and choroid of porcine eyes and with primary porcine RPE cells and then construct a pharmacokinetic model of melanin binding with STELLA® software and simulate it with the *in vitro* results. The compounds chosen for the study; nadolol, timolol, chloroquine, methotrexate, carboxydichlorofluorescein (CDCF) and dexamethasone, are small molecules with diverse physicochemical properties (octanol/water partitioning coefficient (logP), pK_a, acid/base status). Some are also efflux substrates. The *in vitro* binding with melanin was studied at pH 7.4 and in addition at pH 5 for the acidic compounds, since the pH inside melanosomes where melanin is located is acidic. Porcine RPE cells were used to study the amount of uptake and rate of elimination of the set of compounds. The effect of efflux was also evaluated with a general efflux inhibitor probenecid.

All the basic compounds bound to melanin *in vitro*. The acidic compounds did not seem to bind at pH 7.4 but bound at pH 5. Chloroquine, as expected, had the highest binding. In the cell studies, the uptake of chloroquine was significant, at least partly due to melanin binding. The other compounds were taken into the cells to a much smaller extent. The efflux inhibitor did not seem to affect the results. The results of the binding study were used in the models constructed of melanin binding and cellular pharmacokinetics. The constructed model was a very simple one not taking into account many factors affecting cellular pharmacokinetics. The results of both the *in vitro* studies and the model give a good idea of the importance of melanin binding in ocular drug delivery. The model can be used in the future as a base for more comprehensive models of the effect of melanin binding on ocular pharmacokinetics.

Avainsanat – Nyckelord – Keywords

Ocular pigment, melanin binding, *in vitro* studies, pharmacokinetic modeling

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Lääkeaineiden sitoutuminen silmän melaniiniin: *in vitro* –kokeet yhdistettynä farmakokineettiseen malliin

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Tiivistelmä/Referat – Abstract

Lääkeaineet voivat kerääntyä pigmentoituneisiin kudoksiin sitoutumalla solujen sisältämään melaniinipigmenttiin. Melaniini vaikuttaa lääkeaineen farmakokinetiikkaan toimimalla varastona, josta lääkeaine annostelun päättymisen jälkeen vapautuu. Sitoutuminen voi myös aiheuttaa haittoja suuren paikallisen pitoisuuden vuoksi. Tämä tutkimus käsittelee silmän melaniinia. Kirjallisuuskatsauksessa tutustutaan tavallisimpiin melaniinisitoutumisen tutkimusmenetelmiin ja painotetaan *in vitro* sitoutumiskokeita ja tulosten sopivuutta farmakokineettiseen mallinnukseen.

Kokeellisen tutkimuksen tavoitteena oli tutkia muutaman yhdisteen sitoutumista sian silmän verkkokalvon pigmenttiepiteelistä (RPE) ja suonikalvosta eristettyyn melaniiniin sekä lääkeaineiden kinetiikkaa sian RPE:n primaarisoluilla ja yhdistää tulokset STELLA® -ohjelmalla rakennettuun kineettiseen malliin melaniinisitoutumisesta. Tutkittavaksi valittiin yhdisteitä, joilla on erilaiset fysikokemialliset ominaisuudet (oktanoli/vesi -jakautumiskerroin (logP), pK_a, happo/emäs). Osa oli myös efluksitransporttereiden substraatteja. Kaikkien yhdisteiden melaniinisitoutuminen tutkittiin pH:ssa 7,4 ja happamilla molekyyleillä lisäksi pH:ssa 5, sillä melanosomit eli melaniinia sisältävät soluelimet ovat sisällöltään happamia. Solukokeissa mitattiin soluun menevää ainemäärää ja eliminaatiota soluista. Efluksitransporttereiden vaikutusta tutkittiin yleisellä inhibiittorilla (probenesidi).

Kaikki emäksiset yhdisteet sitoutuivat melaniiniin. Happamat yhdisteet eivät sitoutuneet pH:ssa 7,4, mutta sitoutuivat pH:ssa 5. Klorokiini sitoutui odotetusti parhaiten. Solukokeissa se oli myös ainut yhdiste, jota meni merkittävästi solun sisään. Tämä johtui osittain melaniinisitoutumisesta. Efluksi-inhibiittori ei näyttänyt vaikuttavan tuloksiin. Sitoutumiskokeen tuloksia käytettiin rakennetussa farmakokineettisessä mallissa. Mallit rakennettiin sekä melaniinisitoutumisesta että solutason kinetiikasta. Solumalli oli erittäin yksinkertainen, mutta sitä voidaan hyödyntää rakennettaessa kattavampia malleja melaniinisitoutumisen vaikutuksesta lääkeaineen farmakokinetiikkaan silmässä. Tutkimus kokonaisuudessaan osoitti melaniinisitoutumisen tärkeyden lääkeaineiden annostelussa silmään.

Avainsanat – Nyckelord – Keywords
Silmän pigmentti, melaniinisitoutuminen, *in vitro* -kokeet, farmakokineettinen mallinnus

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ABBREVIATIONS

AMD	age related macular degeneration
APS	aminopropyl silica
BCRP	breast cancer resistance protein
BN	brown Norway (rat)
$CaCl_2$	calcium chloride
CDCF	carboxydichlorofluorescein
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic
DMSO	dimethyl sulfoxide
DOPA	dihydroxyphenylalanine
DPBS	Dulbecco's phosphate buffered saline
DT	integration interval (STELLA® software)
D-MEM	Dulbecco's modified eagle medium
EDTA	ethylenediaminetetraacetate
FBS	fetal bovine serum
HPLC	high performance liquid chromatography
IPE	iris pigment epithelium
logP	octanol/water partitioning coefficient
$MgCl_2$	magnesium chloride
MRP	multidrug resistance protein
MTX	methotrexate
PBS	phosphate buffered saline
PET	positron emission tomography
pRPE	porcine retinal pigment epithelium
P-gp	P-glycoprotein
QSPR	quantitative structure-property relationship
QSRR	quantitative structure-retention relationship
RPE	retinal pigment epithelium
SPECT	single photon emission computed tomography
TLSER	theoretical linear solvation energy relationship

UPLC	ultra-performance liquid chromatography
UV	ultraviolet
WBA	whole body autoradiography

1 INTRODUCTION

The accumulation of certain drugs to pigmented tissues has been acknowledged for long (Potts 1962; Potts 1964a). This accumulation is caused by the binding of drugs to melanin, a macromolecule inside pigmented cells. Pigmented cells are found in many tissues of the body including the skin, hair, the brain, the inner ear, and the eye. Melanin affects the pharmacokinetics of a drug by retaining the drug inside pigmented cells and by releasing it when dosing has ended. Thus the binding is a factor to be considered in drug delivery. This thesis will concentrate on ocular melanin binding and ocular drug delivery.

In the eye, melanin containing tissues include the iris, ciliary body, sclera, choroid and retinal pigment epithelium (RPE) (Ings 1984; Durairaj et al. 2012). Ocular drug delivery can be implemented via various routes depending on the target tissue (Figure 1). In diseases of the anterior part of the eye, the drug is most commonly delivered topically as eye drops or formulations with a more prolonged action (Urtti 2006). From the surface of the eye the drug can penetrate the corneal epithelium to reach the aqueous humor (Figure 1 (1)) and then the iris and ciliary body, which are melanin containing tissues. It can also penetrate the conjunctival epithelium and the sclera to reach the ciliary body (2) and come to contact with melanin again. Reaching the posterior part of the eye with topical administration is difficult. When the target tissue is in the posterior part, the drug can be delivered periocularly, by injecting or implanting the drug onto the surface of the sclera (3). In that case, the drug has to penetrate the sclera and depending on the target tissue the ciliary body or the choroid and the RPE, all containing melanin. RPE and retinal vascular endothelium (capillaries) are cell layers with tight junctions and are part of the blood-retinal-barrier, thus constituting a strong barrier for drug delivery. The drug also needs to cross this barrier when it is delivered to the eye systemically (4). Another method to deliver the drug to the posterior part is intravitreal injection. This is the most common route for treating posterior part diseases. The drug is distributed from the vitreous to the surrounding ocular tissues and has again access to melanin in the RPE, ciliary body and even iris. Therefore it can accumulate into these

tissues. The highest melanin content of ocular tissues is in the RPE-choroid and it poses a significant barrier and reservoir for ocular drugs (Menon et al. 1992).

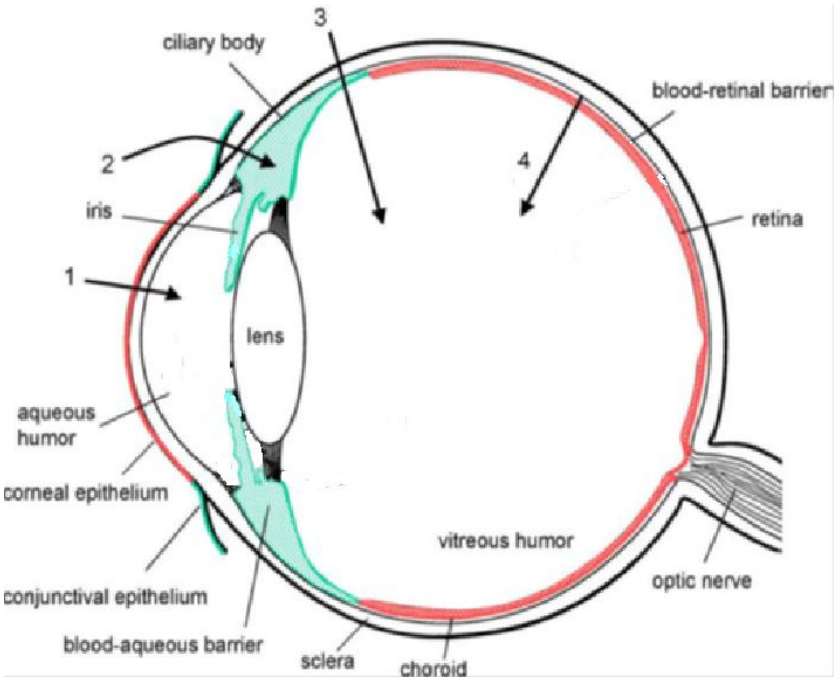

Figure 1. Routes and barriers of ocular drug delivery: topical administration (1, 2), periocular administration (3) and systemic delivery (4). In addition, intravitreal injection, where the drug is administered into the vitreous humor, can be used. (Modified from Urtti 2006)

Melanin binding of drugs has been studied *in vivo* and *in vitro* (Ings 1984). The extent of melanin binding of many ocular drugs has been investigated and some quantitative structure-property relationship models of melanin binding have been created but pharmacokinetic modeling of melanin binding has never been done. The existing pharmacokinetic models of ocular drug delivery do not take into account the effect of melanin binding in detail (Ranta and Urtti 2006; Ranta et al. 2010). Melanin binding is included into the models only via its contribution to measured parameters like the lag time in permeability of melanin containing tissue layers but no individual parameters for melanin binding have been addressed. Creating a pharmacokinetic model of melanin binding and including it into a model of ocular drug delivery would be beneficial for drug discovery as well as a better understanding of the effect of melanin on ocular pharmacokinetics.

2 MELANIN

2.1 Melanin synthesis

Melanin is a polyanionic polymer derived from the amino acid tyrosine mainly via enzymatic and spontaneous reactions (Prota 1980; Ings et al. 1984). There are two types of melanins; eumelanin and pheomelanin. They have different molecular structures and are different in color; eumelanin is brown or black and pheomelanin is yellow or red. Tyrosine is oxidized by the enzyme tyrosinase to dopaquinone, which is the final common precursor of the two melanin types (Figure 2). Eumelanins consist of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) derived subunits, which are formed spontaneously from dopaquinone. Pheomelanins consist of sulfur containing benzothiazine derivatives that are formed when cysteine or other thiol compounds are present to react with the dopaquinone to form cysteinyldopa (Prota 1980; Ito and Wakamatsu 2003). Although multiple enzymes are involved in melanin synthesis, only tyrosinase is essential for the process.

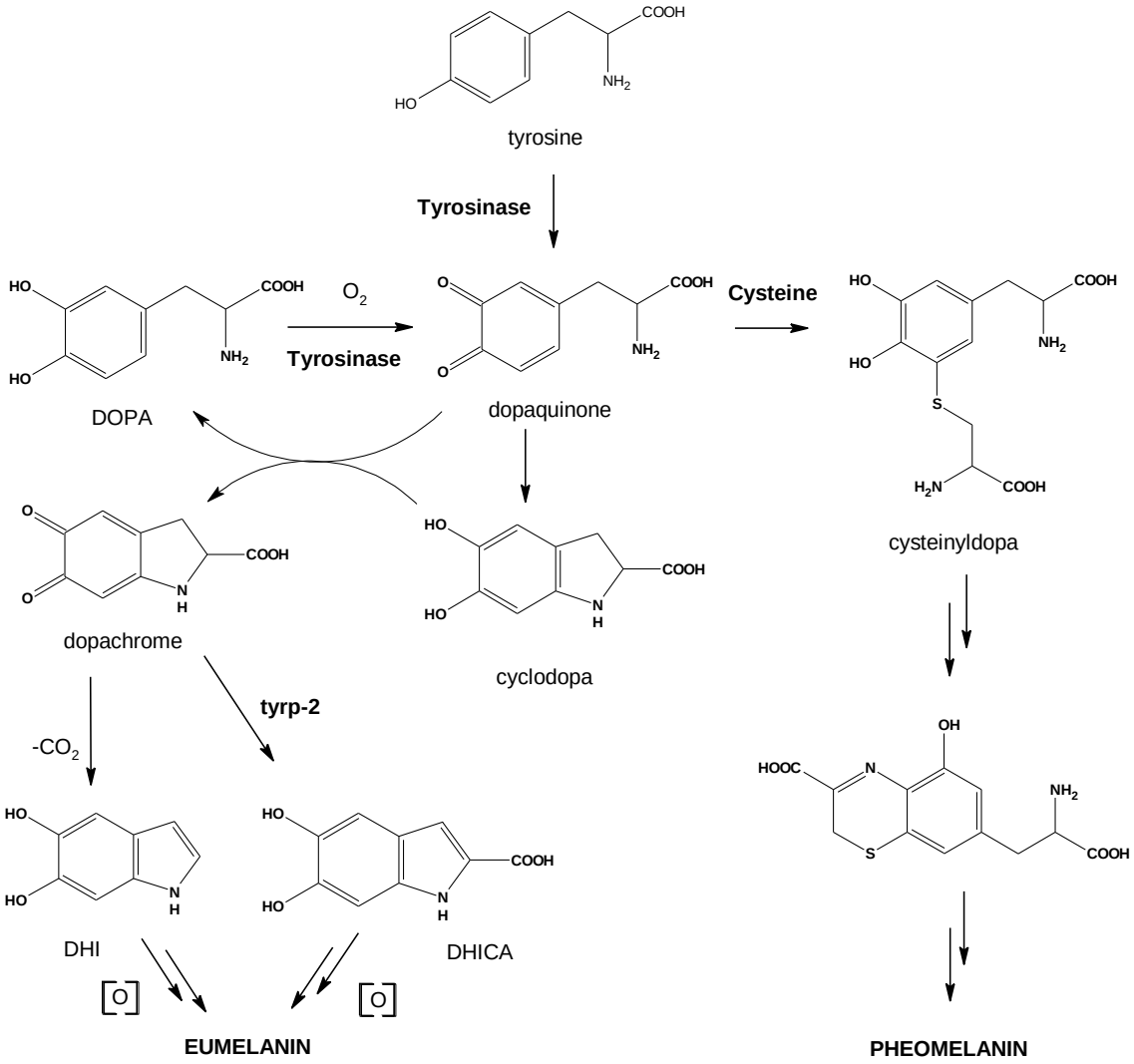

Figure 2. The biosynthesis of melanin from tyrosine or DOPA (modified from Ito et al. 2011).

After the formation of dopaquinone, melanogenesis can be divided into three subsequent stages (Figure 3) (Ito and Wakamatsu 2008). The first stage is the formation of cysteinyldopa in the presence of cysteine. The second stage is the oxidization of cysteinyldopa to pheomelanin. The last stage is the formation of eumelanin. The last step happens when the concentrations of cysteine and cysteinyldopa are sufficiently low for the first and second steps to stop. Thus the ratio of pheomelanin and eumelanin production depends on the activity of tyrosinase and the availability of tyrosine and

cysteine. In a low cysteine concentration, the first and second stages are insignificant, and the last stage leading to eumelanin production is predominant.

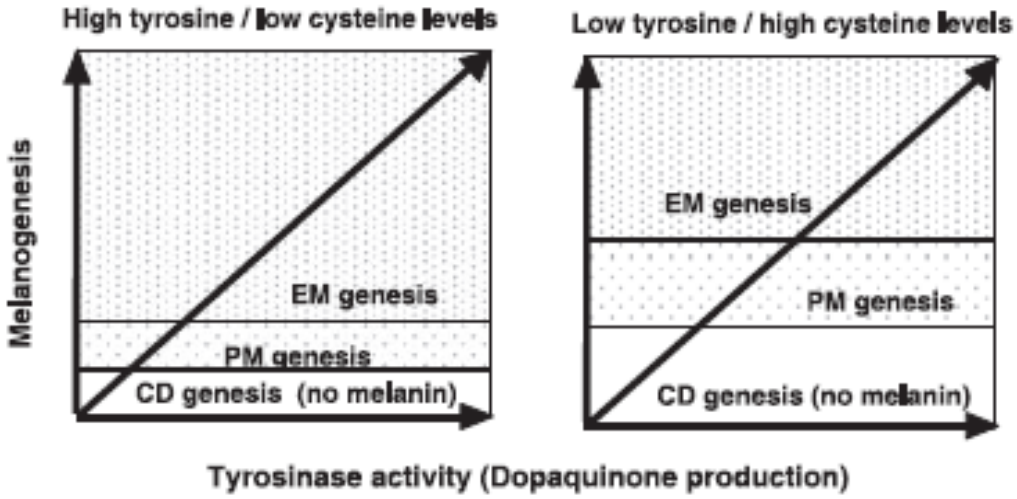

Figure 3. Three stages of melanogenesis. Eumelanogenesis is favored when tyrosinase activity is high and cysteine levels are low (left). Pheomelanogenesis is favored when tyrosinase activity is low and cysteine levels are high. CD=cysteinyldopa, PM=pheomelanin, EM=eumelanin. (Ito and Wakamatsu 2008)

Melanin is synthesized in melanosomes; lipid membrane covered intracellular organelles that contain melanogenic enzymes and cofactors (Kushimoto et al. 2001). Melanosome containing cells are called melanocytes. After the earliest stages of maturation, melanosomes have an organized, fibrillar matrix where the synthesized melanin will bind. Melanin is synthesized as long as there is active tyrosinase in the melanosome. When the melanosome is full, the tyrosinase activity ceases, ending the melanin synthesis.

The pH of melanosomes affects the ratio of eumelanin and pheomelanin formation by affecting tyrosinase activity and the rate of reactions in the first and second stages (Figure 3) (Ancans et al. 2001; Ito and Wakamatsu 2008). At a neutral pH, eumelanogenesis is favored and at acidic pH more pheomelanin is formed. Tyrosinase is most active in near neutral pH and the activity ceases with decreasing pH. Low tyrosinase activity leads to low dopaquinone levels, which favors pheomelanogenesis. The cyclization of cysteinyldopa (stage two) leading to pheomelanin formation is faster

in a low pH and the cyclization of dopaquinone to cyclodopa (stage three) leading to eumelanin formation is slower. This also favors pheomelanogenesis. Higher tyrosinase activity in near neutral pH increases eumelanogenesis.

The pH of most melanosomes is acidic (Bhatnagar et al. 1993; Puri et al. 2000; Ancans et al. 2001). Bhatnagar et al. (1993) determined the melanosomal pH of B16 murine melanoma to be between 3.0 and 4.6. Others have determined the pH of melanosomes in the skin to be acidic as well (Puri et al. 2000; Ancans et al. 2001). Fuller et al. (2001) however noticed a difference in the pH of melanosomes from Caucasian and black skin. While the pH of Caucasian skin melanosomes was acidic, the pH of black skin was more neutral. The pH of ocular melanosomes has not been studied, but it is expected to be acidic. The turnover of melanin and the activity of tyrosinase in ocular melanosomes are low, suggesting a low melanosomal pH (Hu et al. 2008). Detailed studies of the pH of ocular melanosomes are needed.

2.2 Melanin in ocular tissues

In the eye, melanin is found primarily in the uvea and retinal pigment epithelium (RPE), but the sclera also contains melanin (Figure 4) (Ings et al. 1984; Hu et al. 2008; Durairaj et al. 2012). The RPE continues on the surface of the uvea as ciliary and iris pigment epithelia (IPE). Other uveal melanocytes are located in the stromas of the iris and ciliary body and in the choroid. Melanocytes in ocular pigment epithelia (i.e. RPE, IPE and ciliary pigment epithelium) are of different embryonic origin than other ocular melanocytes. Melanocytes in these pigment epithelia are derived from the neural ectoderm (near the optic cup) whereas other ocular melanocytes are derived from the neural crest.

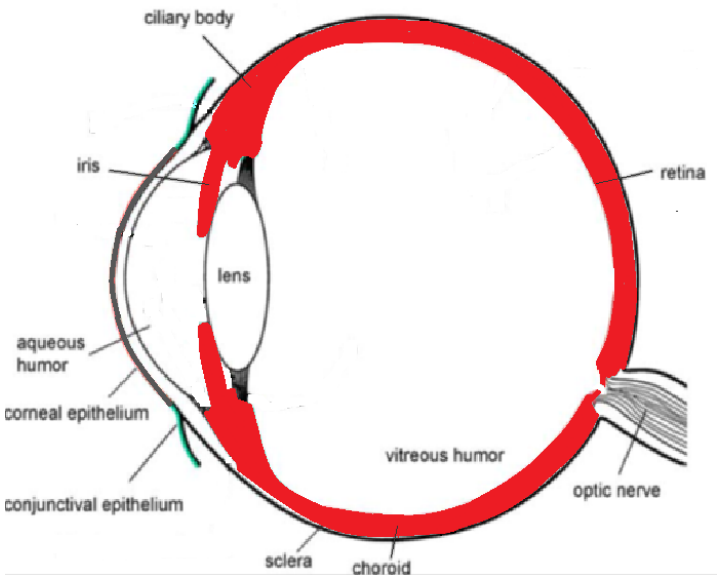

Figure 4. Location of melanin in the eye (red): retina, choroid, sclera, iris, and ciliary body.

2.2.1 Retinal pigment epithelium

The formation of melanosomes in the RPE takes place early during fetal development and melanin synthesis continues until approximately two years of age (Hu et al. 2008). It is not known whether there is any melanin turnover in mature human RPE cells but since aging causes the melanin content of these epithelial cells to decrease significantly, melanin turnover is either very slow or entirely absent (Schmidt and Peisch 1986; Hu et al. 2008).

The RPE is densely pigmented and contains mainly eumelanin (Hu et al. 2008). The amount of melanin in the RPE does not vary significantly with race or iris color (Weiter et al. 1986; Wakamatsu et al. 2008). Melanin is distributed in the RPE so that there is a peak of melanin density at the macula, but around the macula the density is the lowest and again increases towards the equator (Weiter et al. 1986). The amount of melanin decreases with age with a largest decrease in peripheral (close to the equator) melanin. The average amount of melanin in the combined RPE-choroid is approximately 7.5 mg per eye (Table 1) (Menon et al. 1992).

In addition to eumelanin and pheomelanin, the molecular composition of melanosomes includes proteins and lipids. Azarian et al. (2006) identified 102 proteins in porcine RPE melanosomes. These are proteins that are involved for example in melanogenesis, organelle acidification, proteolytic enzymes, and transporters and channels. Ward and Simon (2007) analyzed the lipid composition of bovine ocular melanosomes. They found that there are significant differences between melanosomes in the RPE and uvea. The major component of uveal melanosomes is sphingomyelin while glycerophosphoethanolamine is the most abundant species in RPE melanosomes. The functional role of melanosomal lipids is however mostly undetermined.

2.2.2 Uveal melanin

As mentioned earlier, uveal melanocytes are divided into epithelial melanocytes and other uveal melanocytes. Uveal epithelial melanocytes (ciliary epithelium and IPE) are similar to the RPE and contain mostly eumelanin (Prota et al. 1998). Other uveal melanocytes can be further categorized in choroidal, iridial and ciliary melanocytes. According to Prota et al. (1998) and Wakamatsu et al. (2008) iridial melanocytes contain both pheomelanin and eumelanin and the amount and ratio of these melanin types in the iris determines the color of the eye. Eyes with dark colored irides have a larger amount of eumelanin in uveal melanocytes and a larger eumelanin/pheomelanin ratio than eyes with light colored irides. In light colored eyes the eumelanin/pheomelanin ratio is smaller but the amount of pheomelanin is similar. Wielgus and Sarna (2005) argued that a minimal fraction (a few percent) of iridial melanin is pheomelanin. They however used the iris as a whole (both iridial melanocytes and IPE). Since the IPE contains mostly eumelanin and is much more densely pigmented than iridial melanocytes, the contribution of iridial melanocytes is left minimal (Wakamatsu et al. 2008). Menon et al. (1992) found no significant difference in the amount of melanin in the iris of brown and blue eyes when the iris was again used as a whole. The average amount of melanin in the iris was 2.1 mg (Table 1).

The color of the iris can be expected to affect the extent of melanin binding because of differences in the amount of melanin. Menon et al. (1992) found no difference in the

amount of timolol bound to iridial melanin from blue and brown eyes *in vitro*. However, indications of differences in binding have been obtained *in vivo* (Salminen et al. 1985). Salminen et al. studied the effect of timolol on the intraocular pressure in humans with blue or brown eyes. There was no reduction in intraocular pressure in the brown colored eyes but in blue colored eyes the reduction was significant. The main cause for this difference was concluded to be pigment binding in the iris.

Choroidal melanin is also mainly eumelanin (Wakamatsu et al. 2008). Melanin density in the choroid is the highest at the macula and decreases towards the equator (Weiter et al. 1986). There is a statistically significant difference in the melanin content of the choroid Caucasian and black eyes with black eyes having more melanin than Caucasian eyes.

Table 1. Total amounts of melanin and melanin types (eumelanin or pheomelanin) in ocular tissues.

Tissue	Amount of melanin/tissue (mg)	Melanin type (Eu/Pheo)
RPE	7.5[1]	mainly eumelanin[2]
Choroid		mainly eumelanin[2]
Iris	2.1[1]	2-50 % pheomelanin*[2]
IPE		mainly eumelanin[2]

*depends on the color of the eye
[1](Menon et al. 1992), values for RPE + choroid and iris + IPE
[2](Prota et al. 1998)

2.2.3 Species variation

There are some comparative studies on the species variation of the type of ocular melanin and the content of melanin in different parts of the eye. Liu et al. (2005) studied the type of melanin in the choroid, RPE and iris of mature and newborn bovine eyes. They found that all of these tissues contain mainly eumelanin with pheomelanin content being very low in the choroid and RPE (0.1-0.5 %) and moderate in the iris (< 2 %). Kadam et al. (2011) studied the melanin content of the sclera and choroid-RPE of albino and pigmented rabbits, pigs, cows, and brown Norway (BN) rats. Melanin content in the

sclera was rather low in all species. Melanin content in the choroid-RPE was in the order of BN rat > porcine ~ bovine > pigmented rabbit > albino rabbit. The melanin content of human choroid-RPE reported by Menon et al. is between that of the pigmented and albino rabbits (Menon et al. 1992; Kadam et al. 2011). Durairaj et al. (2012) studied the differences of melanin content in various ocular tissues in humans, minipigs (two strains), rabbits (two strains), dogs and monkeys. In the central choroid-RPE all other species had significantly higher melanin contents than humans. In the peripheral choroid-RPE the case was the same except for the other minipig strain that had equal melanin content to humans.

There are some studies that compare isolated ocular melanin from different species to synthetic melanin by analyzing particle size and surface area (Koeberle et al. 2003; Pitkänen et al. 2007). Pitkänen et al. found that the particle size of isolated bovine melanin and synthetic melanin in a buffered saline solution were similar after the solution was sonicated. Synthetic melanin tended to aggregate more vigorously during storage but aggregation happened also with bovine melanin. The specific surface area of bovine melanin was smaller than that of synthetic melanin. Koeberle et al. (2003) compared isolated bovine and *Sepia* melanin and synthetic melanin. They found that synthetic melanin had a smaller particle size than *Sepia* melanin. Bovine melanin had a wide size distribution with both smaller and much larger particles than synthetic and sepia melanin.

2.3 Functions of ocular melanin

All of the functions melanin may have are not entirely known. In the eye, the absorption of light is thought to be the most important function (Sarna et al. 1992; Hu et al. 2008). Melanin absorbs near infrared light, visible light, and ultraviolet (UV) radiation. In the iris it controls the visible and UV light entering the eye and protects the eye of their harmful effects. In the RPE melanin protects the neural retina from getting exposed to too much light, minimizing reflection and improving image quality.

Melanin acts as an antioxidant and protects the eye against free radicals. This may help protect against diseases such as uveal melanoma and age related macular degeneration (AMD) (Hu et al. 2008). Eumelanin is shown to be more antioxidative than pheomelanin although both melanin types act as antioxidants. There is a higher incidence of AMD in people with light-colored eyes; this is believed to be related to lower amounts of eumelanin in the uvea. Eumelanin, as being also the more photoprotective species, protects uveal melanocytes against malignant changes causing uveal melanoma. On the other hand, constant exposure of melanocytes to oxidizing agents (free radicals, light) in the aging eye may reduce the antioxidant effect of melanin and melanin may even act as a pro-oxidant (Sarna 1992; Hu et al. 2002).

Melanin is known to bind many chemicals, protecting the eye from their harmful effect (Larsson 1993). Among these are free radical forming heavy metal ions like iron and copper and harmful organic compounds, for example drugs. By binding chemicals melanin can also act as reservoir. Depending on the nature of the chemical, this effect can be harmful, if it causes the chemical to accumulate to melanocytes.

2.3 Binding of drugs to melanin

Melanin binding of drugs is widely known (Potts 1964a; Ings et al. 1984; Larsson 1993). The exact mechanism of binding, however, remains undetermined. As previously mentioned, melanin is a polyanionic compound. Electrostatic interactions are thought to be the main contributing mechanism of binding (Ings et al. 1984; Larsson and Tjälve 1979; Lowrey et al. 1997). Other mechanisms include van der Waals forces and hydrophobic interactions. Lowrey et al. (1997) developed a TLSER (theoretical linear solvation energy relationship) model to convey the binding in relation to molecular descriptors of a set of drugs. The model suggested that the interaction was electrostatic in nature. Chloroquine seemed to be an exception in the model, hinting that electrostatic forces are not the main mechanism in its binding. Larsson and Tjälve (1979) as well as Stepien and Wilczok (1982) have also suggested that non-electrostatic interactions are important in chloroquine-melanin binding and that the binding of chloroquine may even be partly irreversible. Van der Waals forces were suggested to be the main contributing

interaction in the binding of chloroquine. Thus it is evident that different mechanisms contribute to drug-melanin binding depending on the nature of the drug.

There is a relationship between the physicochemical properties of a drug and its binding to melanin (Ings 1984; Zane et al. 1990; Leblanc et al 1998). All basic and lipophilic drugs can be expected to bind to melanin (Leblanc et al. 1998). This is expected, since the melanin polymer is polyanionic. Ings (1984) found a good correlation between melanin binding and the pK_a of benzodiazepines. Zane et al. (1990) found a good correlation between the acid/base status, pK_a and octanol/water partitioning coefficient (logP) of a drug and its retention in ocular melanin. Others have confirmed these correlations (Kadam and Kompella 2010).

By binding drugs, melanin can act as an intracellular drug reservoir. This can lower the free, i.e. effective, concentration of the drug inside the cell and thus affect drug response. Binding can also protect the cell from toxic effects of the drug by limiting the intracellular free drug concentration. Studies have shown that certain drugs have harmful effects in the eyes of albino rats but not in pigmented rats (Leblanc et al. 1998). Melanin can, however, also accumulate the drug in melanin containing tissues, like the eye, and expose the melanin containing cell to toxic effects of the drug. Although melanin binding may play a role in the toxicity of a drug to certain tissues, the binding itself does not indicate that a drug is toxic (Leblanc et al. 1998).

Since the amount of eumelanin in ocular tissues is much larger than that of pheomelanin, eumelanin can be considered to be quantitatively more important in drug binding. It has been shown, however, that both melanin types bind drugs (Mårs and Larsson 1999). Mårs and Larsson determined pheomelanin binding of six compounds known to bind to eumelanin. The studies were performed with synthetic pheomelanin. All of these compounds bound to pheomelanin but mainly to a lesser extent. The binding to pheomelanin varied between 29-98 % of the binding to eumelanin (Table 2).

Table 2. Comparative results of pheomelanin and eumelanin binding. I*n vitro* studies were performed with synthetic pheomelanin and bovine ocular eumelanin (Mårs and Larsson 1999).

Compound	Binding (%)		Binding ratio
	Pheomelanin	Eumelanin	Pheo/Eu
Acridine orange	98	100	0,98
Clomipramine	23	80	0,29
Chloroquine	27	92	0,29
Chlorpromazine	36	86	0,42
Nickel	47	88	0,53
Paraquat	22	77	0,29

7 ml of 2.5 µM solution of the compound was incubated with 5 mg of the melanin in question.

3 METHODS FOR STUDYING MELANIN BINDING

Ocular melanin binding has been studied with various methods including *in vitro* methods with isolated or synthetic melanin; *ex vivo* methods with isolated ocular tissues and *in vivo* methods with pigmented and albino animals (Potts et al. 1964a; Ings et al. 1984; Menon et al. 1989). Binding studies concerning other than ocular melanin are similar to the methods used in ocular studies and *in vitro* studies with isolated or synthetic melanin are relatable to melanin binding in other tissues than the eye (Bathory et al. 1987; Larsson 1993; Karlsson et al. 2009). These methods concerning other tissues are not discussed here in detail.

3.1 *In vitro* studies with melanin

The simplest way to study melanin binding of drugs is with isolated or synthetic melanin. This method involves adding a compound solution to a melanin suspension, incubating and separating the insoluble melanin and bound drug from the solution (Potts 1964a). These melanin binding studies have been done since Albert Potts introduced this simple method to study the binding in 1964. Potts first used isolated bovine uveal melanin and synthetic DOPA-melanin to study the interaction of chlorpromazine with melanin pigment (Potts 1964a). In the same number of the same journal he published

results of the recovery of chlorpromazine and chloroquine from isolated bovine choroidal melanin with an alkaline alcohol solution (Potts 1964b). Since then numerous binding studies have been performed with different compounds using melanin from different sources.

3.1.1 Binding parameters of *in vitro* studies

Most *in vitro* binding studies present a maximum binding capacity (B_{max}, nmol/mg of melanin) and an equilibrium dissociation constant (K_d, µM) to depict the binding (Aula et al. 1988; Koeberle et al. 2003; Pitkänen et al. 2007; Pescina et al. 2012). Instead of equilibrium dissociation constant, equilibrium affinity constant is also used (Wilczok et al. 1990). This is the reciprocal of K_d. These parameters have been calculated either by fitting the results to a Langmuir binding isotherm or by Scatchard analysis.

Melanin binding is treated as being analogous to the adsorption of a drug on a solid (Cheruvu et al. 2008). This means using type I binding isotherms, in which the binding reaches a plateau when a monolayer is formed. The Scatchard method compares the amount of bound ligand to the ratio of the amount bound and the free concentration of the ligand (Equation 1; Figure 5). It is a linear representation of the Langmuir binding isotherm (Equation 2).

$$\frac{B}{[L]} = \frac{-B}{K_d} + \frac{B_{max}}{K_d} \qquad (1)$$

where B is the amount of bound ligand (nmol/mg of melanin), B_{max} is the maximum binding capacity (nmol/mg), $[L]$ is the free ligand concentration (µM) and K_d is the equilibrium dissociation constant (µM).

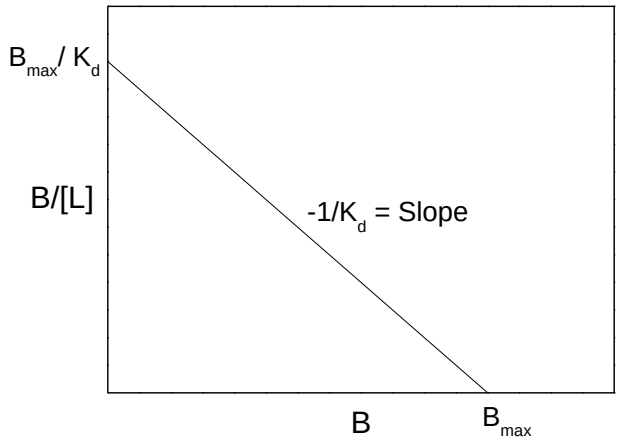

Figure 5. Scatchard plot. B_{max} = maximum binding capacity (nmol/mg), K_d = equilibrium dissociation constant, B = bound amount (nmol/mg), [L] = free ligand concentration (µM).

Linear representations of binding equations are outdated, since more accurate nonlinear curve fitting programs have become available (Invitrogen 2006). A more common way to calculate binding parameters nowadays is nonlinear curve fitting to the Langmuir binding isotherm (Equation 2). This is a better method compared to the Scatchard method, since in the Scatchard method the axis values are not independent as the bound amount (*B*) is featured in both x and y axes (Invitrogen 2006).

$$B = \frac{B_{max}[L]}{K_d + [L]} \qquad (2)$$

There are also other binding isotherms that could be used. Bridelli et al. (2006) compared the fit of different binding isotherms to binding results obtained with three drugs with different physicochemical properties. In addition to the Langmuir isotherm, the results were fit to the Freundlich (Equation 3), Temkin (Equation 4) and Dubinin-Radushkevich isotherms (Equation 5). These isotherms are based on different assumptions; the Langmuir isotherm assumes that adsorption happens at homogenous sites as a formation of a monolayer on a surface, the Freundlich isotherm is used for heterogeneous sites; the Temkin isotherm considers the effects of indirect adsorbent-adsorbate interactions on binding isotherms, and the Dubinin-Radushkevich isotherm expresses adsorption onto a heterogeneous surface. The studied drugs were gentamicin,

methotrexate, and chlorpromazine. Gentamicin was best fitted to the Freundlich isotherm, methotrexate to the Langmuir and Dubinin-Radushkevich isotherms, and chlorpromazine to the Langmuir and Temkin isotherms. Methotrexate and chlorpromazine fitted well to all the isotherms ($R^2 \geq 0.95$) (Table 3). Gentamicin had a very poor fit to the Dubinin-Radushkevich isotherm, but somewhat good fits to the others ($R^2 \geq 0.93$). Gentamicin is the most hydrophilic of the three drugs, methotrexate is somewhat hydrophilic, and chlorpromazine is lipophilic. Thus it can be concluded that the physicochemical properties affect the mechanism of interaction with melanin and could be considered in the analysis of the binding data. However, conclusions cannot be made of the suitability of a certain isotherm to a drug with certain physicochemical properties since the results for the different drugs compared were obtained with different types of melanin (synthetic, bovine and melanoma melanin), which can affect the results as well.

Freundlich isotherm: $$B = K_f * L \quad (3)$$

Temkin isotherm: $$B = \frac{RT}{b} \ln(AL) \quad (4)$$

Dubinin-Radushkevich isotherm: $$B = B_{max} * e^{-\beta \varepsilon^2} \quad (5)$$

where B is the bound amount (mol/g), K_f is a the Freundlich constant (l/g) estimating the adsorption capacity, L is the free concentration of the ligand at equilibrium (mol/l), R is the gas constant (8.314 J/mol/K), T is the temperature (K), b is the Temkin isotherm constant, A is the Temkin equilibrium constant (l/g), B_{max} is the maximum binding capacity (mol/g), β is a constant related to sorption energy and ε is the Polanyi potential.

Table 3. Correlation values for binding results of gentamicin, methotrexate, and chlorpromazine fitted to four different type I binding isotherms (Bridelli et al. 2006).

Drug	**Gentamicin**	**Methotrexate**	**Methotrexate**	**Chlorpromazine**
Melanin type	Synthetic	B16 melanoma	Synthetic	Ocular (bovine)
Correlation coefficients (R^2)				
Langmuir	0,95	0,995	0,997	0,98
Freundlich	0,98	0,95	0,95	0,94
Temkin	0,93	0,98	0,99	0,98
Dubinin-Radushkevich	0,66	0,99	0,997	0,96

Other binding isotherms than the Langmuir isotherm are rarely if ever used in melanin binding studies. The results by Bridelli et al. (2006) suggest that there might not be a need to consider using other isotherms, since all drugs in the study fitted well to the Langmuir isotherm. The Langmuir isotherm is also the simplest considering the parameters it provides. In addition, gentamicin, that had the poorest fit to the Langmuir isotherm, is a very hydrophilic drug, which is rarely the case with new drugs. Therefore the Langmuir isotherm is the most rational isotherm choice.

Bridelli et al. (2006) also studied the association kinetics of melanin binding. The results for the association of gentamicin were fitted to pseudo-first (Equation 6) and second-order kinetic equations, to an intraparticle diffusion model and to the Elovich equation (Equation 7). The best fits were achieved with the Elovich equation and first-order equation. The results did not fit to the pseudo-second-order equation. The first-order equation gives a parameter that is the most straightforward to interpret, since this model gives only one rate constant that is directly related to the differences in the amounts bound at different time points. This model would be the simplest to use in pharmacokinetic models of melanin binding. In a pseudo-first order reaction, the concentration of the other interacting species, in this case melanin, is assumed to stay constant (i.e. very high compared to the other species), thus this concentration is included in the rate constant according to Equation 8.

$$\frac{dB_t}{dt} = k_1 * (B_e - B_t) \quad (6)$$

$$\frac{dB_t}{dt} = a * e^{-bB_t} \quad (7)$$

$$k_1 = k * [M] \quad (8)$$

where B_t (mg/g) is the amount bound at time t (min), B_e is the amount bound at equilibrium (mg/g), k_1 is the pseudo-first-order association rate constant (min^{-1}), a (mg/g/min) and b (g/mg) are Elovich equation constants, corresponding to the rate of sorption and the extent of surface coverage, respectively, k is the actual association rate constant ($min^{-1}M^{-1}$), and [M] is the concentration of binding sites on melanin (M).

The Scatchard analysis, mentioned earlier, is used also for the evaluation of whether there are separate binding site classes for the binding ligand on melanin (Figure 6) (Aula et al. 1989; Invitrogen 2006; Pitkänen et al. 2007). A linear Scatchard plot indicates one binding site class. A concave-up Scatchard plot can indicate two binding site classes or negative cooperativity in binding. In melanin binding studies where Scatchard analysis has been performed, the concave-up curve has been taken as an indication of two binding site classes. The Scatchard plot can also be a concave-down curve, indicating positive cooperativity. This kind of binding has not been met with melanin.

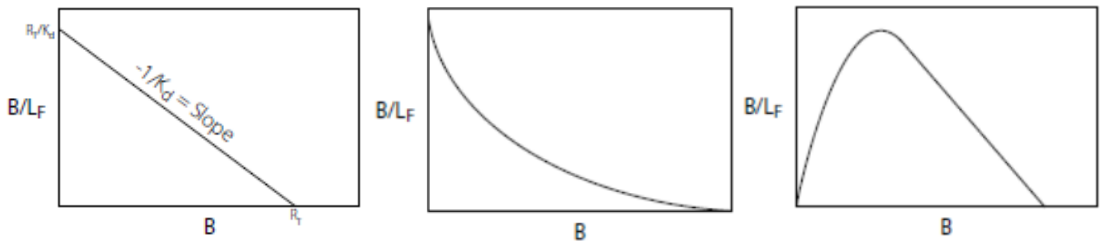

Figure 6. Scatchard plots. A linear plot indicates one binding site class (left), a concave-up curve two binding site classes (middle), and a concave-down curve positive cooperativity. B is the bound amount of ligand, L_F the free ligand concentration, K_d the equilibrium dissociation constant and R_T the total amount of binding sites (the same as the maximum binding capacity, B_{max}). (Invitrogen 2006)

Association to melanin is known to be rather fast, happening within hours (Aula et al. 1988; Pitkänen et al. 2007). There are only a few studies reporting values for

association rate constants (Aula et al. 1988; German et al. 1999). German et al. (1999) studied the association of five antimuscarinic drugs, including for example atropine, from *Sepia* melanin. They found the dissociation to be biphasic with significantly differing fast and slow phases. The association happened within 2 hours. They reported association rate constants of 287 x 10^6 $\mu M^{-1}min^{-1}$ and 1.43 x 10^6 $\mu M^{-1}min^{-1}$ for the fast and slow phase, respectively, for atropine. These values seem surprisingly large, since for example in the case of receptor binding, where association happens within 10 minutes, the rate constants are smaller. Thus an error has most likely occurred in the calculation. Aula et al. (1988) studied the association of timolol to bovine ocular melanin and also got two association rate constants, 5.95 $\mu M^{-1}min^{-1}$ and 0.492 $\mu M^{-1}min^{-1}$. The association happened within five hours. These values seem more reasonable.

There is rather limited information in the literature about the dissociation of drugs from melanin. Aula et al. (1988; 1989) studied the dissociation of timolol and prostaglandin $F_{2\alpha}$ from bovine ocular melanin. They found the dissociation to be linear with dissociation rate constants of 0.0051 min^{-1} and 0.025 min^{-1}. German et al. (1999) also studied the dissociation of five antimuscarinic drugs. They found the dissociation to be monophasic, with a rate constant of 0.01 min^{-1} for atropine. The other drugs had smaller dissociation rate constants, the smallest being 0.003 min^{-1} for tropicamide.

The dissociation rate from melanin can be considered a more important parameter than the association rate, as it reveals how long the drug would stay melanin bound and is released from melanin after dosage. In the few studies on this issue, dissociation has been a slower process than association, thus affecting the time course of melanin binding more significantly (Aula et al. 1988; German et al. 1999).

3.1.2 Melanin sources for *in vitro* studies

The most common sources for isolated melanin used in binding studies on ocular melanin are bovine and porcine eyes and *Sepia officinalis* (cuttlefish) (Potts 1964a; Ings 1984; Koeberle et al. 2003; Buszman et al. 2008). In addition, synthetic melanin is also

used. Synthetic melanin is prepared from dihydroxyphenylalanine (DOPA) or tyrosine via oxidation by an oxidizing agent, e.g. hydrogen peroxide, or by tyrosinase (Potts 1964a; Sigma-Aldrich 2014b). Synthetic and Sepia melanin are available also commercially (Sigma-Aldrich 2014b). Synthetic melanin differs structurally from natural (isolated) melanin (Nosfinger et al. 2000). Nosfinger et al. studied the structures of synthetic and *Sepia* melanin (eumelanin) by scanning electron microscopy. They came to the conclusion that natural eumelanin has a larger size-to-volume ratio than synthetic melanin. This means there are more reactive sites on natural melanin per weight unit of melanin, which can affect the protective properties of melanin as well as the capacity of compound binding. One of the reasons they proposed for this difference was the different molecular structures of these melanin types. As eumelanin is constituted of DHI and DHICA-derived units, synthetic melanin is mainly formed from DHI-derived units.

Melanin binding has been studied in many reports and several studies have included the same drugs in their test repertoire. There are however only a few comparative studies with melanin from different sources (Potts 1964a; Aula et al. 1989; Koeberle et al. 2003; Pitkänen et al. 2007; Cheruvu et al. 2008). These studies have mostly compared an isolated natural melanin to synthetic melanin; only Koeberle et al. (2003) compared two natural melanins, *Sepia* and bovine ocular melanin. In most of the studies (except for Potts (1964a) and Cheruvu et al. (2008)), there were differences in the results obtained with different melanins. Koeberle et al. (2003) studied only the binding of memantine and found that *Sepia* and synthetic melanins had a maximum binding capacity of 68 and 54 % of that of bovine ocular melanin, respectively, when studied in phosphate buffered saline (PBS) (Table 4). Affinities of binding were similar with *Sepia* and synthetic melanin but bovine ocular melanin only had an affinity of half of the values of the other melanins. They also found differences in the binding parameters when the interaction was studied in deionized water instead of saline. One of the reasons for this difference is metal ions in the saline solution binding to melanin and decreasing the ability of other compounds to bind (Wrzesniok et al. 2012). In deionized water, *Sepia* melanin had the highest binding capacity and synthetic melanin the smallest, with affinities being similar with all melanins (Koeberle et al. 2003). This is an

indication of differing structures of these melanins and differences in the results caused by the study environment. Pitkänen et al. (2007) compared bovine choroid-RPE melanin to synthetic melanin with two beta-blockers, betaxolol and metoprolol. They found that synthetic melanin bound significantly more drug per mass unit of melanin. When the results were normalized to the surface area of melanin the differences were much smaller. Cheruvu et al. (2008) studied the binding of celecoxib to synthetic and *Sepia* melanin but found no significant difference in binding. They did not perform a surface area measurement, thus the relation of binding to surface area was left unclear.

Table 4. Results of melanin binding studies comparing melanin from different sources.

Drug	**Parameters**	**Melanin source**		
		Sepia	**Synthetic**	**Bovine ocular**
Koeberle et al. (2003)				
Memantine	B_{max} (nmol/mg)	140	111	207
(PBS)	K_d (μM)	452	442	804
Memantine	B_{max} (nmol/mg)	466	272	364
(deionized water)	K_d (μM)	435	488	351
Pitkänen et al. (2007)				
Betaxolol	B_{max} (nmol/mg)	-	0.152 / 36.3[1]	0.013 / 27.5
	K_d (μM)	-	0.642 / 97.6	0.026 / 249
Metoprolol	B_{max} (nmol/mg)	-	0.166 / 40.7	0.096 / 26.9
	K_d (μM)	-	0.915 /172	0.832 / 495
Aula et al. (1989)				
Prostaglandin $F2_{\alpha}$	B_{max} (nmol/mg)	-	0.0034 / - [2]	0.0025 / -
	K_d (μM)	-	0.0050 / 0.19	0.0037 / 1.99

[1]Pitkänen et al. (2007) reported values for two binding site classes.
[2]Aula et al. (1989) reported a maximum binding capacity (B_{max}) only for the high affinity site.

The largest differences in binding with melanin from different sources have been obtained with methotrexate. There are studies where methotrexate bound to melanin at neutral or near neutral pH and studies where no binding happened (Tsuchiya et al. 1987; Hayasaka et al. 1988; Wilczok et al. 1990). Wilczok et al. (1990) studied the interaction with synthetic melanin and melanin from mouse B16 melanoma at pH 7.0. They found noticeable binding to both melanin types. Tsuchiya et al. (1987) studied the interaction with bovine ocular acid-insoluble melanin at pH values of 4.8, 7.4 and 8.0. Methotrexate did not bind at the higher pH values but at pH 4.8 melanin binding was seen. Hayasaka et al. (1988) obtained similar results as Tsuchiya et al. (1987) by

studying methotrexate binding to synthetic melanin at pH 4.8 and 7.0. The difference caused by the change in pH could be expected since methotrexate is a dicarboxylic acid with pK_a values of 4.1 and 3.4. At the lowest pH, part of methotrexate is in the neutral form that is expected to bind better to melanin than the negatively charged form of methotrexate. This is explained by the polyanionic nature of melanin. The differences in binding at the near neutral pH are more curious. A possible explanation for these differences is the sources of melanin used, although deviant results were also obtained in the two studies done with synthetic melanin. There were no significant differences in other study conditions. Comparative studies should be performed to investigate the cause of this kind of a difference.

Since studies have shown that there are differences in binding between synthetic and isolated melanin and even isolated natural melanin from different sources, the comparison of results from different studies and with different melanin sources should be done carefully. In most binding studies a surface area measurement of melanin has not been performed, which makes direct comparison difficult. Surface area is an important factor to consider since it directly affects the binding sites available for the binding ligand. If the results are needed only to see if a drug binds to melanin, the source of melanin does not seem to affect the conclusion. When more accurate results are required, the effect of melanin source on the results becomes more significant. More information is needed on the relation of binding and surface area and also the comparison of results from different binding studies.

3.1.3 Affinity chromatography

Synthetic melanin has also been used in melanin binding studies done with high performance liquid chromatography (HPLC) (Ibrahim and Aubry 1995; Knörle et al. 1998). Synthetic L-DOPA melanin has been covalently linked to an aminopropyl silica (APS) stationary phase or formed *in situ* with the APS (Ibrahim and Aubry 1995). Ibrahim and Aubry (1995) studied the retention of five phenothiazines and determined binding parameters (maximum binding capacity and affinity) for two of those. When compared to literature results obtained with synthetic and bovine ocular melanin, the

results of the maximum binding capacity were similar, but the affinity to the stationary phase melanin was significantly lower. They concluded possible reasons for the difference to be the immobility of melanin and the different type of melanin used. Despite the differences, the use of this HPLC method could provide a simple and rapid method for studying melanin binding. Aubry (2002) later reviewed the advantages and disadvantages of this method, concluding it to be a useful tool to obtain comparative results with a set of compounds studied by this method. More information is needed however of the relation of these results to the binding study results obtained with the more common *in vitro* method addressed earlier.

The investigations of melanin binding of drugs *in vitro* in early drug discovery would greatly benefit of methods suitable for high throughput screening. In addition to the affinity chromatographic method with HPLC, a magnetic bead method has been introduced (Marszall et al. 2011). In this method synthetic melanin was immobilized covalently to the surface of magnetic iron beads with a diameter of 1 µm. After the incubation of these melanin coated beads with the study compounds (phenothiazines), the supernatant with the free compound was removed with a magnetic separator for analysis. The melanin-covered beads were then washed by a special method and reused. The authors concluded this method to be suitable for high throughput screening and they were able to create a quantitative structure-property relationship (QSPR) model of calculated molecular descriptors related to the binding efficiency to the melanin coated beads. They suggested this to be a useful method to evaluate the affinity of drug candidates to melanin in early drug discovery. The created model is described more accurately later (see 4. Prediction of melanin binding).

3.2 *In vitro* studies with melanosomes

In addition to *in vitro* binding studies with melanin, binding has been studied with isolated melanosomes (Abrahamsson et al. 1988; Debing et al. 1988; Wilczok et al. 1990). Debing et al. (1988) studied the binding of 15 drugs, including for example chloroquine, chlorpromazine, norepinephrine and nicotine, to bovine ocular melanosomes. All compounds bound and the binding curves seemed similar in shape

with melanin binding studies. No quantitative comparison to melanin binding of these drugs was done. Wilczok et al. (1990) studied the binding of methotrexate to melanosomes and melanin isolated from mouse B16 melanoma. Methotrexate bound to both melanosomes and melanin and no significant difference was found between the bound amounts when the amount bound to melanosomes was normalized to the melanin content inside the melanosomes. Abrahamsson (1988) studied the binding of beta-blockers timolol and H216/44 to bovine ocular melanosomes. They compared the results to chlorpromazine, a high binding drug. Chlorpromazine had the highest binding, as expected, but both beta-blockers also bound. The maximum binding capacities of the beta-blockers were between 10-20 nmol/mg of melanosomes. The results were not compared to melanin binding results and the amount of melanin in the melanosomes was not analyzed. Thus it is difficult to draw conclusions on the correlation of these two study methods. The binding curves however were similar in shape as in melanin binding studies.

The isolation methods of melanosomes have been similar to melanin isolation with a sucrose gradient, but no protease has been used (Abrahamsson 1988; Debing et al. 1988; Wilczok et al. 1990; Pitkänen et al. 2007). Ocular tissues (choroid, iris, ciliary body and RPE) have been homogenized and then centrifuged with a sucrose gradient. This has been a common method for the isolation of melanin granules or melanosomes and separates the melanosomes from other cellular components, including mitochondria (Menon and Haberman 1974; Liu et al. 2005). In melanosome studies, the isolated product has been called either melanin granules or melanosomes. In the studies in which protease digestion has been used, the product has been called melanin granules or melanin. The authors have not commented more thoroughly on the composition of the product. The separation between melanosomes and melanin can thus be difficult. Pitkänen et al. (2007) got similarly shaped granules with protease digestion and subsequent sucrose gradient centrifugation as Liu et al. (2005) without protease digestion (Figure 7). The isolated melanosomes were not characterized in any of the binding studies with melanosomes (Abrahamsson et al. 1988; Debing et al. 1988; Wilczok et al. 1990), thus the quality of the melanosomes and whether they were intact remains undetermined.

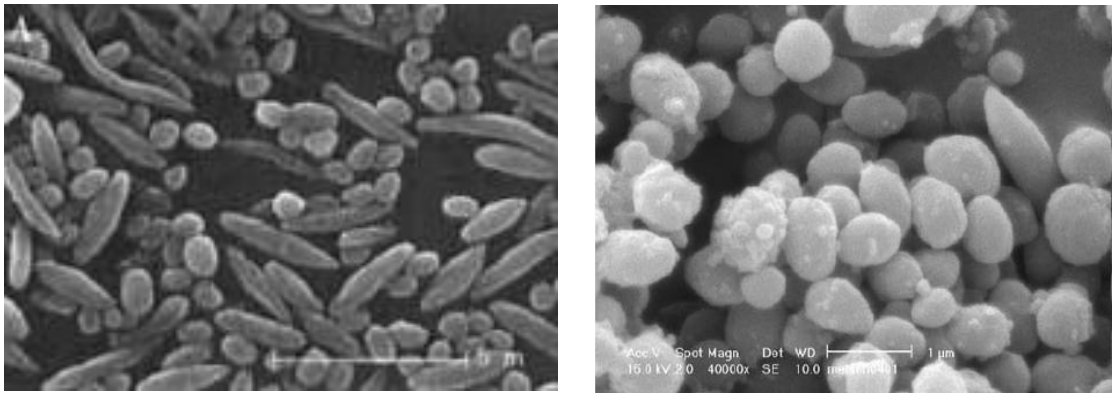

Figure 7. Scanning electron micrographs of bovine ocular RPE melanosomes isolated without protease digestion (left) and bovine RPE melanin granules isolated using protease digestion (right). (Liu et al. 2005; Pitkänen et al. 2007)

Since melanin is inside melanosomes in the cells, it would be beneficial to study the interaction with melanosomes instead of pure melanin. This resembles the *in vivo* situation since the lipid membrane covering the melanosomes affects the ability of drugs to reach the melanin (Larsson 1993). Although a compound may bind to melanin, it might not be able to reach the melanin inside the cells *in vivo*. Since no specific separation of the isolation methods of melanin and melanosomes has been made, the "melanin" in melanin binding studies may be very similar to those studies done with melanosomes. Protease digestion would be expected to deproteinize the granules, thus there may be a differences in the protein composition of the differently isolated granules. However, Larsson and Tjälve (1979) have shown that protein moieties do not affect the binding. Therefore the binding may be very similar in these differently isolated melanin granules. A comparative study would be needed on this matter to better evaluate the effect of isolation methods.

3.3 *Ex vivo* studies

Ocular melanin binding has also been studied *ex vivo* with enucleated eyes, layers of ocular tissue or tissue homogenates. A common *ex vivo* experiment is a permeation study across pigment containing cell layers, like sclera-choroid-Bruch's membrane, isolated from animal eyes (Kadam et al. 2011; Pescina et al. 2012; Du et al. 2013). Kadam et al. (2011) studied the permeation of eight beta-blockers across the sclera, sclera-choroid-RPE and choroid-RPE of albino and pigmented rabbits, pigs and human.

Pescina et al. (2012) studied the permeation of four drugs across pigmented and non-pigmented porcine sclera-choroid-Bruch's membrane. Light blue porcine eyes were used as the non-pigmented control, since in these eyes melanin is only present in the RPE. Du et al. (2013) studied the permeation of triamcinolone acetonide across sclera-choroid-Bruch's membrane of albino and pigmented rabbits. The drawback of the studies by Pescina et al. and Du et al. is the RPE layer being left out of the tissue sample. Since the highest level of melanin in ocular tissues is in the RPE, the study does not take into account the entire barrier in permeation from the sclera border to the vitreous. However, a good estimate of the effect of melanin on the pharmacokinetics can be made, since the pigmented RPE of the light blue eyes does not interfere with the results. This kind of comparative results could be beneficial for a pharmacokinetic compartmental model, since they take into account the differences in the lag time in permeability caused by melanin binding.

Kadam et al. (2011) also studied transscleral transport of eight beta-blockers to the vitreous by measuring the drug levels in different parts of the eye (sclera, choroid-RPE, retina, vitreous) after subconjunctival injection to a euthanized rat and subsequent enucleation of the eye after 6 hours (Kadam et al. 2011). Only pigmented rats were used for this study. Since there was no albino control, an accurate estimate of the effect of pigment could not be made. These results were however used for the assessment of the goodness of correlation of rabbit, porcine and human tissue layer models mentioned earlier. When studying pigment binding, a permeation study would be more rational to perform, since it is not as laborious as a whole tissue *ex vivo* study and the results are comparable.

Menon et al. (1989) studied the melanin binding of timolol with excised and chopped ocular tissues; the iris and ciliary body as pigmented tissues and lens and cornea as non-pigmented controls. The studies were done in a similar manner as *in vitro* binding studies, by letting the chopped tissue interact with timolol in solution. No significant difference was found between the pigmented and non-pigmented tissues when normalized to the wet weight of the tissue. This kind of a study gives a good idea of the binding to different parts of the eye but does not give reasonable estimates of the effect

of pigment binding since a negative non-pigmented version of the same tissue is not used.

3.4 *In vivo* studies

In addition to the methods mentioned earlier, melanin binding can be studied *in vivo* with autoradiography or other imaging methods like positron emission tomography (PET) or single photon emission computed tomography (SPECT) by giving an animal or human a radiolabeled dose of the drug in question. In fact, quantitative autoradiography in rats is often the first indication of melanin binding in drug discovery since binding is not routinely tested with other methods (Solon et al. 2002). Comparative studies can be done with albino and pigmented animals.

There are numerous studies done with whole-body autoradiography (WBA) comparing albino and pigmented mice or rats (Lyden et al. 1982; Larsson et al. 1988; Bathory et al. 1990). A common study design has been to give a single dose of a drug to the animal, then euthanize the animal after a certain time and measure the radioactivity. The desired tissue (e.g. the eye) can also be excised for the measurement. Study time frames have differed from a few hours to three months (Lyden et al. 1982; Bathory et al. 1990). In some studies, an *in vitro* melanin binding study has been performed as well. These kinds of studies give a good idea of the real situation *in vivo* and can help evaluate the relationship between melanin binding and tissue accumulation. However, no numerical relationships have been drawn in any studies to compare the *in vivo* situation to the *in vitro* melanin binding. Other imaging methods, PET and SPECT, have mainly been used so far for the diagnosis of melanoma by melanin binding indicators (Goto 2004; Kato et al. 2006).

In addition to imaging methods, pigment binding has been studied *in vivo* by determining drug concentrations of excised tissues (Sauer and Anderson 1994; Cheruvu et al. 2008). The animal is dosed while alive. After a suitable time, the animal is euthanized and the desired tissue is excised, homogenized and analyzed with a suitable method (e.g. with HPLC). The results obtained of these studies are similar to WBA,

except more accurate concentrations in the tissues and tissue fractions are obtained. Since there is no need for radioactive tracers, this method is simpler compared to imaging methods.

Another way to study pigment binding *in vivo*, and more closely the effect of binding on the effect of a drug, is to study the differences in drug response of pigmented and albino animals. A common way to study drug response in ocular tissues has been to study intraocular pressure after the dosing of pressure decreasing drugs (antiglaucomatics) (Shibata et al. 1988; Nagata et al. 1993). These studies can also be done in humans comparing eyes with brown and blue irides (Salminen et al. 1985). Also miosis and mydriasis have been studied (Salazar et al. 1976; Urtti et al. 1984). While being a valuable tool in the evaluation of differences in drug response caused by pigment binding, these studies lack the possibility of providing accurate data of the quantity of drug bound to melanin *in vivo*.

3.5 Cell models of melanin binding

The results from *in vitro* binding studies are depicting only the interaction between melanin or melanosome and the drug, and do not take into account other processes happening *in vivo*. Studying the interaction with cells could therefore be beneficial. This would take into account some cellular factors, including melanin being inside cells and in the cells inside melanosomes, covered by lipid membranes that restrict access to melanin.

Hornof et al. (2005) reviewed current cell models for ocular barriers, and from this review it is obvious that many cell culture models of the RPE do not take into account the effect of melanin. No literature could be found on melanin binding itself being studied with cells but a number of cell models of the RPE have been created. These cultures have been characterized by their morphology, bioelectric parameters (transepithelial electrical resistance), permeability, and expression of certain proteins. When evaluating melanin binding drugs with these cell models, it would be important to include the characterization of melanin content of the cells.

When studying melanin binding *in vitro* with cells, the cells should naturally be pigmented. Primary RPE cells contain melanin until at least the fourth passage and can be used in these studies (Dorey et al. 1990; Hornof et al. 2005). The use of primary cells however is laborious, since the isolation procedure is time consuming. Immortalized human RPE cells have been shown to become melanized (i.e. synthesize melanin) when kept in confluence for four weeks in serum free conditions or more than eight weeks in serum containing medium (Rambhatla et al. 2002). The use of these cells is however hindered by the long culture duration. There are also reported methods for increasing the melanin content of RPE cells in culture, such as feeding the RPE cultures melanin granules (Boulton and Marshall 1985; Basu et al. 1989). Boulton and Marshall (1985) fed isolated melanin granules to human RPE cells by incubating the cells in growth medium suspended with melanin granules. The granules were taken up by the cells and the cells remained melanized at least for seven days. The authors concluded this method to be a possibility for cell models of RPE that do not contain melanin. Basu et al. (1989) used a similar method successfully, reported in their published abstract. Kadam et al. (2012) developed an MDCK cell line model for the RPE with tunable melanin expression. MDCK cells were transfected by retroviral infection with genes of human tyrosinase and p-protein, a protein shown to play a role in the correct processing and localization of tyrosinase. These cells were compared with MDCK cells without melanin by doing uptake studies with chloroquine, a drug with very high affinity to melanin, and salicylic acid, a low affinity drug. The cell uptake of chloroquine was 2.3-fold higher in pigmented MDCK cells than in non-pigmented cells. No difference was found with salicylic acid. Thus the melanin binding was shown to affect the uptake considerably. This kind of cells could be used in uptake studies of melanin binding and could help solve the problem of the need for primary or other time consuming cultures.

To be able to use cell binding studies in pharmacokinetic modeling of melanin binding, the amount of melanin inside the cells should be measured. The melanin content of cultured primary RPE cells decreases over time (Dorey et al. 1990). Differences in melanin content will also occur when melanin granules are fed to the cells. Thus it is important to quantify the amount of melanin to be able to normalize the results to the amount of melanin. There are reported methods for the analysis of melanin content, the

most used being absorbance measurement of intact or lysed cells (Donatien and Orlow 1995; Rambhatla et al. 2002; Kadam et al. 2012). These methods are not discussed here.

4 PREDICTION OF MELANIN BINDING

As mentioned earlier, melanin binding can be related to the physicochemical properties of the drug. Some quantitative structure property relationship (QSPR) models or similar quantitative models have been constructed (Raghavan et al. 1990; Radwanska et al. 1995; Lowrey et al. 1997). These models are however not very comprehensive, since the sets of drugs used for model construction have been small and somewhat homologous.

All basic and lipophilic drugs can be expected to bind to melanin, since these drugs have the potential to bind to the polyanionic melanin as well as cross the lipid membranes needed to access melanin inside cells. Zane et al. (1990) compared the physicochemical properties of 27 drug candidates to the distribution into the melanin-containing uveal tract of the rat eye determined by whole body autoradiography. The physicochemical properties included were molecular weight (between 223 and 489), pK_a, degree of ionization, logP, drug-melanin binding energy and acid/base status. Distribution was measured at 5 minutes and 96 hours after dosing. At 5 minutes, the primary factors affecting the distribution to the uveal tract were, in decreasing order of effect, acid/base status, pK_a, binding energy, and logP. The retention (96 hour time point) in the uveal tract correlated best with the volume of distribution, logP, pK_a and binding energy. The correlation predicted with these parameters was better in the distribution phase (5 min) than in the retention phase, the values of R^2 being 0.961 and 0.577, respectively. The authors concluded the most notable factor contributing to the poorer correlation at 96 hours to be metabolism. WBA does not differentiate between the drug and the metabolite. No quantitative model was created based on the physicochemical parameters.

The same group calculated drug-melanin binding energies using molecular modeling (Raghavan et al. 1990). They used a 5,6-dioxoindole (a monomer of melanin) as a tetramer as the model for melanin. Binding energies obtained by modeling were then compared with *in vitro* results. The correlation was good, with an R^2 value of 0.811. They concluded that this method can be considered a significant predictor of melanin binding *in vitro*.

The TLSER model (Equation 9) developed by Lowrey et al. (1997) of the equilibrium constant (*K*) of melanin binding was comprised of four prescriptors, "covalent" hydrogen bond basicity, ε_B "covalent" hydrogen bond acidity, ε_A, and electrostatic hydrogen bond basicity, q.. Other descriptors considered for the model but excluded for poor fits, were molecular volume, polarizability index, and electrostatic hydrogen bond acidity, q_+. The R^2 value for the equation was 0.77. 16 compounds were used for the model.

$$logK = 181\ \varepsilon_B + 9.73q_- + 78.4\varepsilon_A - 47.9 \qquad (9)$$

Radwanska et al. (1995) derived a quantitative structure retention relationship (QSRR) model by analyzing the retention of 15 phenothiazines with HPLC reported by Lowrey et al. (1997) and Markuszewski and Kaliszan (2002). They correlated an HPLC capacity factor to drug hydrophobicity determined on an immobilized artificial membrane column and the energy of lowest unoccupied molecular orbital determined by molecular modeling. Lowrey et al. (1997) transformed this QSRR to a TLSER model by substituting the parameters with surface area accessible to water and ε_A. Both of these models can be used to depict the mechanisms involved in binding, as well as to predict the binding of other similar drugs.

As mentioned earlier, Marszall et al. (2011) introduced a magnetic bead method for the studying of melanin binding of drugs and created a QSPR model of the relationship of binding efficiency to melanin and calculated theoretically structural descriptors. These descriptors were calculated from the molecular formula of these compounds and have no definite physical meaning. This model was constructed with 17 similar compounds,

most of which were antipsychotics. It was concluded to describe well the binding efficiency of these compounds to melanin but also the potential of these compounds to cause extrapyramidal syndrome, a condition caused by antipsychotics that has been related to melanin binding in the central nervous system. Thus this model can be used in early drug discovery of this group of drugs. Although other possibilities, e.g. the evaluation of ocular toxicity, were mentioned for this model, the weakness of the model is the small number and similarity of the compounds used.

Predictive models of melanin binding would be beneficial in drug discovery, helping to get an idea of whether melanin binding should be considered, possibly as a disadvantage or even an advantage e.g. in the form of a drug reservoir. Building a QSPR model of melanin binding would constitute a simple method in evaluating melanin binding already in early drug discovery. The weakness of the models built so far is the limited amount of compounds used. Much more extensive data should be used to be able to construct a comprehensive model. Also, pharmacokinetic modeling of melanin binding and its effect on the pharmacokinetics of drugs has never been done. It would be beneficial to include melanin binding to pharmacokinetic models of drug delivery to be able to evaluate the *in vivo* situation already in early drug discovery.

5 AIMS OF THE STUDY

The aims of the experimental part of the study were to evaluate the effect of melanin binding on cellular and ocular pharmacokinetics in the posterior part of the eye, more closely in the retinal pigment epithelium (RPE), and to construct a cellular level pharmacokinetic compartmental model of the binding. These aims were accomplished by:

1. Investigating the melanin binding of a set of compounds *in vitro* with porcine ocular melanin and obtaining parameters depicting the amount and kinetics of melanin binding

2. Investigating the cellular pharmacokinetics of this set of compounds with porcine RPE (pRPE) cells and obtain parameters of cellular pharmacokinetics.
3. Evaluating the effect of RPE efflux transporters (MRP1, MRP4 and MRP5) in the cell studies with a general efflux inhibitor probenecid
4. Constructing a pharmacokinetic model of melanin binding and simulating it with the binding parameters obtained from *in vitro* studies
5. Combining the binding model to a cell model and modeling the effect the binding has on cellular pharmacokinetics.

6 MATERIALS AND METHODS

6.1 Materials

Nadolol and chloroquine diphosphate salt were obtained from Sigma-Aldrich (St. Louis, MO, USA). Timolol, carboxydichlorofluorescein (CDCF), methotrexate, dexamethasone and probenecid were all provided by The University of Helsinki. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was used as a solvent for the highest concentrations of stock solutions, which were then diluted with phosphate buffered saline (PBS) (pH 7.4, without $CaCl_2$ and $MgCl_2$) (Gibco, Invitrogen, NY, USA) or citrate buffer (pH 5) (APPENDIX 1).

Cells were cultured in Dulbecco's modified eagle medium (D-MEM) 31885 (Gibco, Invitrogen, NY, USA) supplemented with 10 % fetal bovine serum (FBS), 50 U/ml penicillin and 50 U/ml streptomycin. Cells were detached with 0.25 % trypsin-EDTA (Gibco) and washed with DPBS (with or without $CaCl_2$ and $MgCl_2$).

6.2 *In vitro* binding studies with melanin

6.2.1 Isolation of melanin granules

The melanin used in the binding studies was isolated from the RPE and choroid of porcine eyes. Fresh porcine eyes were obtained from a slaughter house (HK Ruokatalo, Forssa) and kept on ice during the transport. Extraocular material was cleaned from the eyes with scissors, the eyes were dipped in ethanol and then in PBS before the eyes were cut (first batch 51 eyes, second batch 26 eyes). The eyes were cut circumferentially behind the limbus and the anterior part of the eye with the vitreous was gently removed. The remaining eye cup was turned inside out. The outermost cell layer (neural retina) was gently removed uncovering the RPE and the choroid. The RPE-choroid was separated from the sclera, placed in PBS (pH 7.4) and stored at -20 °C until melanin isolation.

Melanin isolation from the RPE-choroids was modified from Pitkänen et al. (2007) and Pescina et al. (2012). Subtilisin protease type VIII from Bacillus licheniformis (Sigma-Aldrich, St. Louis, MO, USA) was added to the RPE-choroids in PBS, with the amount of the protease being at least 30 mg/25 eyes. The suspension was incubated at 56 °C for one hour with manual stirring every 10 minutes and then heated to 95 °C for 15 minutes to inactivate the protease. The suspension was centrifuged at 37 000 g for 15 minutes and the supernatant was discarded. The pellet was washed with PBS and centrifuged as above. The suspension was vacuum filtered on a Büchner funnel through filter paper. The filtrate was centrifuged at 37 000 g for 15 minutes and the precipitate was mixed with Milli-Q water and lyophilized overnight.

6.2.2 Characterization of melanin granules

Particle size of the melanin granules was measured with Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Larger aggregates were evaluated with a light microscope (Leica DM IL LED inverted microscope, Leica microsystems, Wetzlar, Germany) and images taken with Leica EC3 camera. Particle size measurements were

done in PBS at pH 7.4. Samples were prepared as in the binding study (see 6.2.3 Binding study) and incubated in the same conditions for 0, 3, 5 or 20 hours. Since some particles were too large for measurement with the Zetasizer, all samples were filtrated with a 5 µm pore size Ultrafree-MC centrifugal filter (Millipore, Billerica, MA, USA). After filtration, the samples were diluted 1:10 to a 0.1 mg/ml melanin concentration.

Zeta potential of the melanin granules was measured with the Zetasizer Nano ZS. Since there is an expected difference in zeta potential at different pH values, measurements were done in both experimental pH environments, pH 5 (in citrate buffer) and pH 7.4 (in PBS). Both batches of isolated melanin were measured. 1 mg/ml melanin suspensions were made in both citrate buffer and PBS to mimic the experimental conditions of the binding studies. The suspensions were warmed to 37 °C, sonicated for 15 minutes, and diluted to a 0.2 mg/ml concentration for the measurement.

6.2.3 Binding study

Melanin binding studies were performed with six compounds; nadolol, timolol, chloroquine, carboxydichlorofluorescein (CDCF), methotrexate and dexamethasone. Stock solutions were made in DMSO (nadolol, timolol, CDCF, dexamethasone), Milli-Q water (chloroquine) or 0.1 M sodium hydroxide (methotrexate). In the final solutions used in the binding experiments, DMSO concentration did not exceed 2 %.

Melanin binding was studied with all the compounds at pH 7.4 and in addition at pH 5 with methotrexate and CDCF. Methotrexate and CDCF are acidic compounds, the pK_a values for methotrexate are 4.1 and 3.4 (a dicarboxylic acid) and for CDCF 5.1 (Table 5). The other compounds are basic except for dexamethasone which is a very weak acid (pK_a 11.4). Experiments at pH 7.4 were done in PBS and at pH 5 in citric acid buffer.

Table 5. Physicochemical properties and transporter interaction of the compounds studied.

Compound	logP	logD (7.4)	pK_a	Acid/base	Efflux substrate
Timolol	1.8[1]	0.34[1]	8.9[1]	base	P-gp
Nadolol	0.93[1]	-0.82[1]	9.2[1]	base	no
Chloroquine	3.7[2]	1.9[3]	8.1 and 10.4[2]	base	MRP1[6], P-gp
Methotrexate	- 0.45[3]	-4.9[3]	4.1 and 3.4[4]	acid	MRP1-4, -8, BCRP, P-gp etc.
CDCF	2.6[3]	-0.50[3]	5.1[5]	acid	MRP5[7]
Dexamethasone	1.9[2]	1.9[3]	11.4[4]	acid	P-gp

[1](Kadam and Kompella 2010)
[2](Verbeeck et al. 2005)
[3]values predicted with ACD/Labs (Chemspider 2014)
[4]values predicted with ChemAxon (Chemspider 2014)
[5](Sigma-Aldrich 2014a)
[6](Vezmar and Georges 1998)
[7](Mannermaa et al. 2009)
If not otherwise indicated, efflux substrate information from TP-Search: Transporter Database (2014).

Four types of binding studies were performed; kinetic studies to determine how long it takes for the binding to reach equilibrium; equilibrium studies to determine the maximum binding capacity (B_{max}) and equilibrium dissociation constant (K_d); dissociation study to determine the rate constant for dissociation from melanin (k_{off}), and association study to compare the calculated value of the association rate constant (k_{on}) to the measured value (Table 6).

Melanin suspension and test compound solutions were prepared right before every experiment. Freeze dried melanin was mixed with PBS or citrate buffer to form a 2 mg/ml suspension. The suspension was sonicated for 15 minutes before incubation with the test compounds. Test compound solutions were prepared from 50 mM stock solutions stored at -20 °C.

Kinetic studies were performed with nadolol, timolol, methotrexate, dexamethasone and CDCF at a melanin concentration of 0.5 mg/ml and with nadolol and chloroquine at 1 mg/ml. CDCF was studied at 0.1 µM concentration and the other compounds at 1 µM, due to limitations of the analysis method, ultra performance liquid chromatography (UPLC). 75 µl of compound solution was added to 75 µl of melanin suspension (1 mg/ml or 2 mg/ml) in eppendorf tubes and incubated in a shaker (220 rpm) at 37 °C. The time points were 1, 3, 5 or 6 and 21 hours, with three replicates for each time point. Control samples (three replicates) were incubated in PBS without melanin. After the

incubation, the eppendorf tubes were centrifuged at 17 000 g for 15 minutes. The supernatant was collected for UPLC analysis.

Equilibrium binding studies with a concentration range of 0.25-500 µM (n=11) were performed for all the compounds. The binding was measured at a time point where equilibrium of binding was reached. 20 hours was chosen due to experiment scheduling and to guarantee the equilibrium state. The concentration of the prepared melanin suspension was 2 mg/ml. 70 µl of compound solution was added to 70 µl of melanin suspension, and incubated as in kinetic studies for 20 hours. Controls were prepared with 70 µl of PBS instead of melanin suspension. Number of replicates was three. The experiments were performed on Skirted 96 well PCR plates with removable 8-strip Flat Caps (4titude, Wotton, Surrey, UK). After the incubation the well plates were centrifuged at 1800 g for 4 minutes, to remove the condensed water from the caps to the solution in the wells. The contents of the wells were then moved to eppendorf tubes and treated as previously in the kinetic study.

Dissociation studies were performed at 1 µM and 10 µM concentrations of nadolol, timolol and chloroquine at a melanin concentration of 1 mg/ml. 50 µl of compound solution was added to 50 µl melanin suspension (2 mg/ml) in an eppendorf tube. The binding was allowed to reach equilibrium, after which the melanin-compound mixture was diluted 1:20 with PBS to initiate dissociation. Incubation conditions were the same as in previous experiments. Dissociation of bound compound into the PBS solution was measured until 24 hours (n=9) and for chloroquine also at 120 hours (5 days). 100 µl samples were taken at each time point.

An association study was performed with nadolol at concentrations 1 and 10 µM. 700 µl of compound solution was added to 700 µl of melanin suspension (2 mg/ml) in eppendorf tubes and incubated at 37 °C. 100 µl samples were taken between 15-300 minutes (n=8), with manual shaking of the tube before sample taking. Control samples were incubated without melanin, and samples were taken at 0, 180 and 300 minutes. Number of replicates was again three.

Table 6. Binding studies performed.

	Melanin binding studies				
Compound	**Kinetics**	**Equilibrium**		**Dissociation**	**Association**
	(melanin conc. (mg/ml))	**pH 7.4**	**pH 5**		
Timolol	0.5	yes	-	yes	-
Nadolol	0.5 and 1	yes	-	yes	yes
Chloroquine	1	yes	-	yes	-
Methotrexate	0.5	yes	yes	-	-
CDCF	0.5	yes	yes	-	-
Dexamethasone	0.5	yes	-	-	-

Experimental melanin concentration 1 mg/ml if not otherwise indicated.

6.2.4 Sample analysis

Samples with concentrations over 0.1 µM were analyzed with UPLC (Aquity UPLC, Waters, Milford, MA, USA) with UV detection (Photodiode Array Detector, Waters, USA). The separation was carried out on a UPLC HSS T3 (1.8 µm, 2.1 x 50 mm) column (Waters, USA) at 30 °C. Injection volume was 10 µl. Gradient mode was used for all the compounds with acetonitrile/15 mM phosphate buffer (pH 2) mobile phase. Gradient duration was 3-5 minutes depending on the sample.

For the samples with less than 0.1 µM of the studied compound, a mass spectrometric analysis with UPLC separation was used. The liquid chromatography instrument was Waters Aquity UPLC (Waters, Milford, MA, USA). The separation was carried out on a Waters UPLC HSS T3 (1.8 µm, 2.1 x 100 mm) column at 25 °C. Injection volume was 0.5 µl. Gradient mode was used with acetonitrile (0.1 % formic acid)/water (0.1 % formic acid) mobile phase. For nadolol, the water solvent had no formic acid. Mass spectrometric measurements were carried out using Waters Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization with negative mode for CDCF and positive mode for the other compounds. Propranolol was used as an internal standard.

For the equilibrium study with CDCF, fluorescence analysis was performed on a VarioskanFlash (Thermo Scientific, Waltham, MA, USA) fluorescence plate reader

with 510 nm excitation and 535 nm emission. Samples were diluted 1:10 with 0.1 M sodium hydroxide before analysis.

If not analyzed on the same day, samples were stored at -20 °C until analysis. Samples were also prepared of melanin incubated with PBS (without any test compounds) and treated as test samples to evaluate the effect of melanin on the analyses.

6.2.5 Calculation of the binding parameters

As done in melanin binding studies in general, the binding was assumed to be analogous to the adsorption of a compound on a solid (Pitkänen 2007; Cheruvu et al. 2008). Thus the results of the equilibrium study were fit in a Langmuir binding isotherm for one (Equation 2) and two (Equation 10) binding site classes. The number of binding site classes was evaluated with Scatchard plots, Hill plots and the goodness of fit of the binding isotherm. Nonlinear curve fitting was performed with SigmaPlot software (version 11.0, Systat Software, San Jose, CA, USA)

$$B = \frac{B_{max}[L]}{K_d + [L]} \quad (2)$$

$$B = \frac{B_{max1}[L]}{K_{d1} + [L]} + \frac{B_{max2}[L]}{K_{d2} + [L]} \quad (10)$$

where B is the amount of compound (ligand) bound to melanin (nmol/mg), B_{max} is the maximum capacity for the binding of the ligand (nmol/mg), $[L]$ is the concentration of the free compound (µM), and K_d is the equilibrium dissociation constant (µM).

The value for the dissociation rate constant was calculated according to Equation 11:

$$B_t = B_0 * e^{-k_{off} * t} \quad (11)$$

where B_t is the amount bound (nmol) at time t (h), B_0 is the total amount bound (nmol) at time zero, and k_{off} is the dissociation rate constant (h^{-1}).

Binding was also analyzed with the Hill plot. It is mainly used in receptor binding studies and gives an idea of the number of binding site classes and whether there is cooperativity between binding sites (Invitrogen 2006). The Hill coefficient is the slope of the linear transformation of the Hill equation:

$$log\frac{B}{R_T - B} = n * logL - logK_d \qquad (12)$$

where B is the amount of drug bound to melanin (nmol/mg), R_T is the total amount of binding sites in melanin (nmol/mg), n is the Hill coefficient, L is the free concentration of the drug (µM) and K_d is the dissociation constant (µM). The total amount of binding sites in melanin is not known, so different values starting from the calculated B_{max} values were used. The Hill coefficient values were interpreted according to Table 7.

Table 7. The interpretation of Hill coefficients (Invitrogen 2006).

Hill coefficient	Interpretation
1	One class of binding sites
< 1	One class of binding sites, negative cooperativity
1 < n < 2	One class of binding sites, positive cooperativity or two classes of binding sites, negative cooperativity
2	Two classes of binding sites

6.3 Cell study with porcine RPE cells

6.3.1 Isolation of RPE cells from porcine eyes

The RPE cells used for the cell uptake study were isolated from porcine eyes. Fresh porcine eyes were obtained from a slaughter house (HK Ruokatalo, Forssa). During transport the eyes were kept in ice. Extraocular material was cleaned from the eyes with scissors, the eyes were dipped in ethanol and placed in PBS until all eyes were cleaned. 20 eyes were used to fill one T25 cell culture flask (Sarstedt, Nümbrecht, Germany). Two T25 bottles of cells were used for the study, thus isolation of the cells was done twice. After the cleaning, the eyes were managed in batches of five. The anterior part of

the eye with the vitreous was removed in the same manner as in the isolation of melanin. The eye cup was then filled with PBS (without $CaCl_2$ and $MgCl_2$) which was removed after 10 minutes. The neural retina was carefully removed with tweezers. The eye cups were filled with 0.25 % trypsin-EDTA and incubated for 30 minutes at 37 °C in a humidified atmosphere with 5 % CO_2. The detached cells in the trypsin-EDTA solution were collected into 50 ml centrifuge tubes and twice the volume of the trypsin-EDTA-cell solution of growth medium (10 % (v/v) fetal bovine serum, 1 % penicillin-streptomycin in D-MEM 31885) was added. The tubes were centrifuged three times; first at 450 g for 5 minutes, then twice at 200 g for 2 minutes, in between replacing the supernatant with growth medium. The cells were suspended again in medium and transferred to the cell culture bottle.

6.3.2 Cell culture

Medium for the pRPE cells in the cell culture bottle was changed twice a week. The cells were seeded on a 48-well plate (Nunclon™ Delta surface, 1.1 cm^2/well, Thermo Scientific, Waltham, MA, USA) after two to three weeks in the cell culture bottle. The cell monolayer was washed with pre-warmed DPBS (without $CaCl_2$ and $MgCl_2$). The cells were incubated at 37 °C (5 % CO_2) with 0.25 % trypsin-EDTA for 5 minutes and then suspended in growth medium. Cell density was adjusted to 300 000 cells/ml. 240 µl (ca. 72 000 cells) of cell suspension was added to each well and incubated overnight before the experiments.

Cells in the cell culture bottle were observed with a light microscope (Leica, Wetzlar, Germany) at days 4, 8 and 14 after the cell isolation and 1 day after seeding on the well plate to assure the cells were dividing and contained melanin.

6.3.3 Cell experiments

Compound solutions were prepared aseptically from filter sterilized (Corning® (MA, USA) syringe sterile filter with regenerated cellulose membrane, pore size 0.20 µm) stock solutions. Stock solutions were prepared in DMSO or Milli-Q water (see 6.2.3

Binding study). Final compound solutions were diluted from the stock solutions with D-MEM 31885. The concentration of DMSO in the final solutions did not exceed 1 %.

Uptake experiments were performed with all the compounds at concentrations 1, 5 and 10 µM with an incubation time of 20 hours. At 1 µM concentration of nadolol, timolol, dexamethasone and chloroquine also time points 1 and 5 hours were studied. With efflux substrates chloroquine, methotrexate and CDCF the uptake study was also performed at a 1 µM concentration with a general efflux inhibitor probenecid. Probenecid concentration was 10 µM, enough to assure efflux inhibition (Mannermaa et al. 2009). Time points with the inhibitor were 1, 5 and 20 hours. Elimination studies were performed with chloroquine and timolol without the efflux inhibitor and with chloroquine, methotrexate and CDCF in the presence of the inhibitor. To reach equilibrium before initiating the elimination, the cells were incubated with the compound solutions for 20 hours. Time points for the elimination study were 0.5, 1, 2, 4, 6, 14, and 48 hours. Each experiment was performed in duplicate. Cell studies performed are compiled to Table 8.

Table 8. Cell studies performed.

	Cell studies			
Compound	**Uptake**	**Uptake with inhibitor**	**Elimination**	**Elimination with inhibitor**
Timolol	yes	-	yes	-
Nadolol	-	-	-	-
Chloroquine	yes	yes	yes	yes
Methotrexate	yes	yes	-	yes
CDCF	yes	yes	-	yes
Dexamethasone	yes	-	-	-

Before the experiments, growth medium was removed from the wells and cells were washed with pre-warmed DPBS (with $CaCl_2$ and $MgCl_2$). 250 µl of compound solution was added to the wells. In the inhibitor studies, the cells were pre-incubated with an inhibitor for 15 minutes before adding the compound solution that also included the inhibitor. After the incubation time, the solution in the wells was placed in an eppendorf

tube and centrifuged at 12 000 g for 1 minute. The supernatant was collected for analysis. The cells left in the well were lysed with 150 µl of 0.1 M ammonium hydroxide and the suspension centrifuged at 17 000 g for 5 minutes. The supernatant was collected for analysis. In the elimination studies, at each time point, 250 µl of solution was removed for analysis and replaced with 250 µl of medium (D-MEM 31885). The cells were lysed after the last time point.

Control samples were incubated on the 48-well plates without any cells. Incubation conditions were the same as with the cells. Number of replicates for each sample was two.

6.4 Pharmacokinetic modeling

Pharmacokinetic simulation models were built with STELLA® software (Version 9.0.1, Isee Systems, Lebanon, NH, USA). Runge-Kutta 4 was used as the integration method and the integration interval (DT) was 0.001 h. Melanin binding was assumed to be identical to a bimolecular reaction

$$M + L \leftrightarrow ML$$

where M is free melanin, L is the free binding ligand (drug) and ML is the bound melanin-ligand complex. The rate of the melanin-ligand complex formation is depicted by Equation 13:

$$\frac{d[ML]}{dt} = k_{on} * [M] * [L] - k_{off} * [ML] \qquad (13)$$

where the symbols in square brackets express concentration (nmol), *M* for melanin, *L* for the binding ligand, and *ML* for the melanin-ligand complex. k_{on} is the association rate constant ($nmol^{-1}h^{-1}$) and k_{off} the dissociation rate constant. In the case of the two-site binding model, both sites are treated according to Equation 13 giving the sum

$$\frac{d[ML_1]}{dt} + \frac{d[ML_2]}{dt} = k_{on1} * [M_1] * [L] - k_{off1} * [ML_1] + k_{on2} * [M_2] * [L] - k_{off2} * [ML_2] \qquad (14)$$

In a pharmacokinetic model, the amounts of ligands are used instead of concentrations. Thus both sides of Equation 13 can be multiplied with volume, giving the equation the form

$$\frac{dML}{dt} = k_{on} * [M] * L - k_{off} * ML \quad (15)$$

The association rate constant (k_{on}) was calculated from the equilibrium dissociation constant (K_d) and dissociation rate constant (k_{off}):

$$k_{on} = k_{off}/K_d \quad (16)$$

Both melanin binding and cell uptake together with melanin binding were modeled. The rate of cell uptake was depicted with Equation 17:

$$Rate = P_{app} * SA * (C_L - C_{L,O}) \quad (17)$$

where P_{app} is the apparent permeability of the cell membrane (dm/h), *SA* is the surface area of the cells (dm^2), C_L is the concentration of the free ligand inside the cells (nM) (as *L* in Equations 13, 14 and 15), and $C_{L,O}$ in the concentration of the ligand outside the cells (nM).

Equilibrium binding was modeled with one and two-site binding models for all the compounds that bound to melanin and gave reasonable parameter values with both equations. Association and dissociation from melanin were modeled with different k_{off} values (and calculated k_{on} values, $k_{on} = k_{off}/K_d$) to see how this affects the equilibrium time and dissociation time with the set of studied compounds. The values for maximum binding capacity (B_{max}) and equilibrium dissociation constant (K_d) were obtained from the *in vitro* binding study.

Cell uptake was modeled with the cell study concentrations 1, 5 and 10 µM with and without melanin binding. Values for P_{app} were obtained from literature. Pitkänen et al. (2005) have determined permeability values of RPE-choroid for lipophilic and hydrophilic beta-blockers; 0.0036 dm/h for lipophilic ones and 0.00072 dm/h for

hydrophilic ones. These values were treated as monolayer permeabilities and converted to cell membrane permeability values by multiplying with two. The corresponding P_{app} values for the model were 0.0144 dm/h and 0.00288 dm/h, for lipophilic and hydrophilic drugs, respectively. Chloroquine, timolol and dexamethasone were considered to be lipophilic and nadolol, methotrexate and CDCF as hydrophilic.

The amount of melanin inside the cells was considered to be 7.5 mg in the RPE-choroid based on the analyses of Menon et al. (1992). The volume of the RPE-choroid was assumed to be 10 µl (Ranta et al. 2010). Thus the melanin concentration used in the model was 750 000 mg/l. The volume of the cells was assumed to be 0.1 µl.

7 RESULTS

7.1 Characterization of melanin granules

The melanin granules were weighed after drying. The weights were 98 mg for the first batch (51 eyes) and 30 mg for the second batch (26 eyes). The results of the particle size measurement are presented in Figure 1. The size is reported as an average diameter (µm) and an average polydispersity index. Microscope images showed significant aggregation of melanin granules after 20 h incubation; the largest particles in the 20 h incubated suspension were in the 100 µm range, as the largest particles in the freshly sonicated solution were less than 20 µm and most particles smaller 5 µm (APPENDIX 2). Chloroquine seemed to stabilize the granules when observed with the microscope, thus the particle size analysis with Zetasizer Nano ZS was performed also together with chloroquine. In this analysis there was no significant difference in the particle sizes (Figure 8).

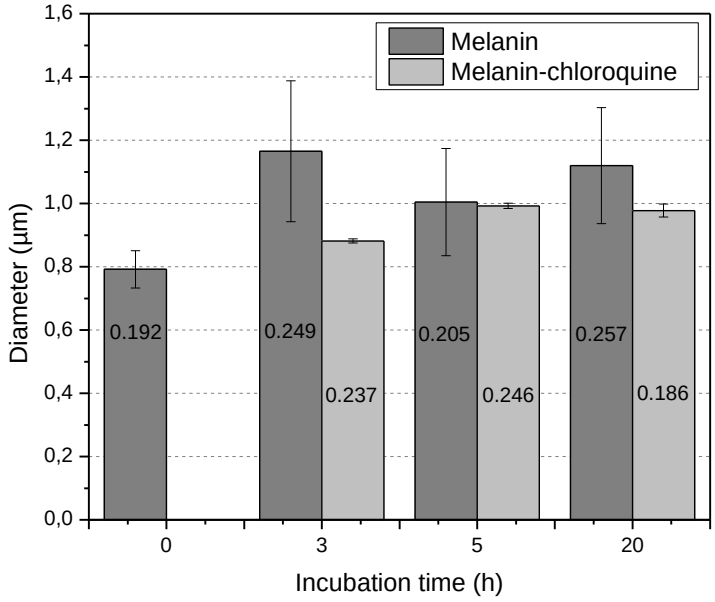

Figure 8. Particle size measurement of 0.1 mg/ml melanin suspension and melanin (0.1 mg/ml) - chloroquine (10 µM) suspension after different incubation times. Polydispersity indices are presented inside the bars. (Samples were filtrated after incubation with a 5 µm filter.)

Zeta potential measurements were done at pH 5 and 7.4 and of both batches of melanin.

Zeta potential was slightly lower (more negative) at pH 7.4 (Figure 9).

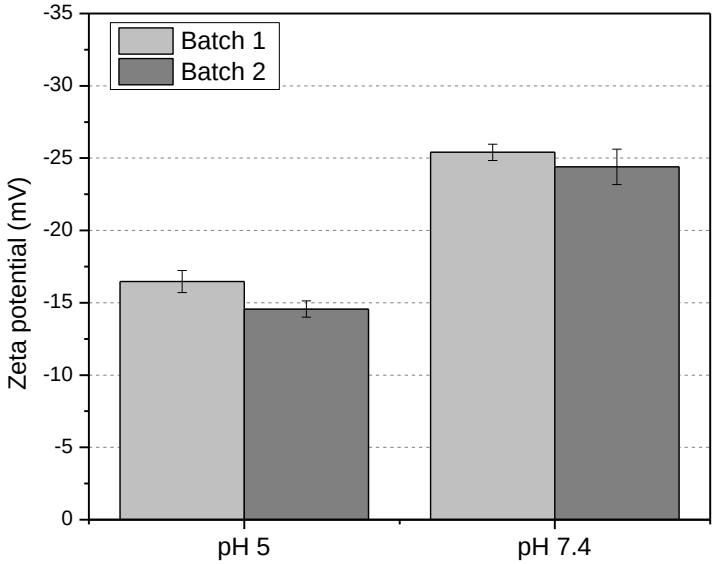

Figure 9. Zeta potentials of the two isolated melanin batches at pH 5 and 7.4 with the standard deviation of three consecutive measurements of the same sample in the parentheses.

7.2 Binding study

7.2.1 Kinetic study

At 0.5 mg/ml melanin concentration the binding of nadolol and timolol had reached equilibrium in 6 hours (APPENDIX 3). Methotrexate did not seem to bind to melanin. With CDCF and dexamethasone the results were inconclusive. Ca. 10 % of CDCF seemed to bind after 20 hours of incubation. Dexamethasone seemed to bind but the time course of binding was unclear. The binding of timolol was between 10-20 % at all the time points. Ca. 10 % of nadolol bound to melanin at 0.5 mg/ml melanin concentration and 14 % at 1 mg/ml melanin concentration. Ca. 99 % of chloroquine bound to melanin. At 1 mg/ml melanin concentration nadolol reached the equilibrium state by the 5 h time point and chloroquine already by the 1 h time point.

7.2.2 Equilibrium study

The results for the equilibrium binding study are presented in Table 9 and Figure 10. Chloroquine had the highest binding, as the kinetic study already showed. Methotrexate did not bind at pH 7.4 but at pH 5 had over 70 % binding at the smallest concentrations. The amount of bound timolol (11-21 %) was similar to that of nadolol (8-24 %). Nadolol had a higher variation in the binding between different concentrations. The results were inconclusive with CDCF at pH 7.4, but if any binding happened, it was very low. At pH 5 there was ca. 30 % binding at the smallest concentrations. Dexamethasone seemed to bind but the binding was rather low, ranging between 4-10 % with no correlation with the starting concentration.

Table 9. Fractions bound to melanin in the concentration range that gave successful results.

Compound	Concentration range (µM)	Fraction bound (%)
Nadolol	0.25-500	8-24
Timolol	1-500	11-21
Chloroquine	0.25-500	50-99.6
Methotrexate (pH 7.4)	0.25-500	0-3
Methotrexate (pH 5)	0.25-500	18-76
CDCF (pH 7.4)	0.25-500	1-8
CDCF (pH 5)	0.25-100	11-30
Dexamethasone	0.25-50	4-10

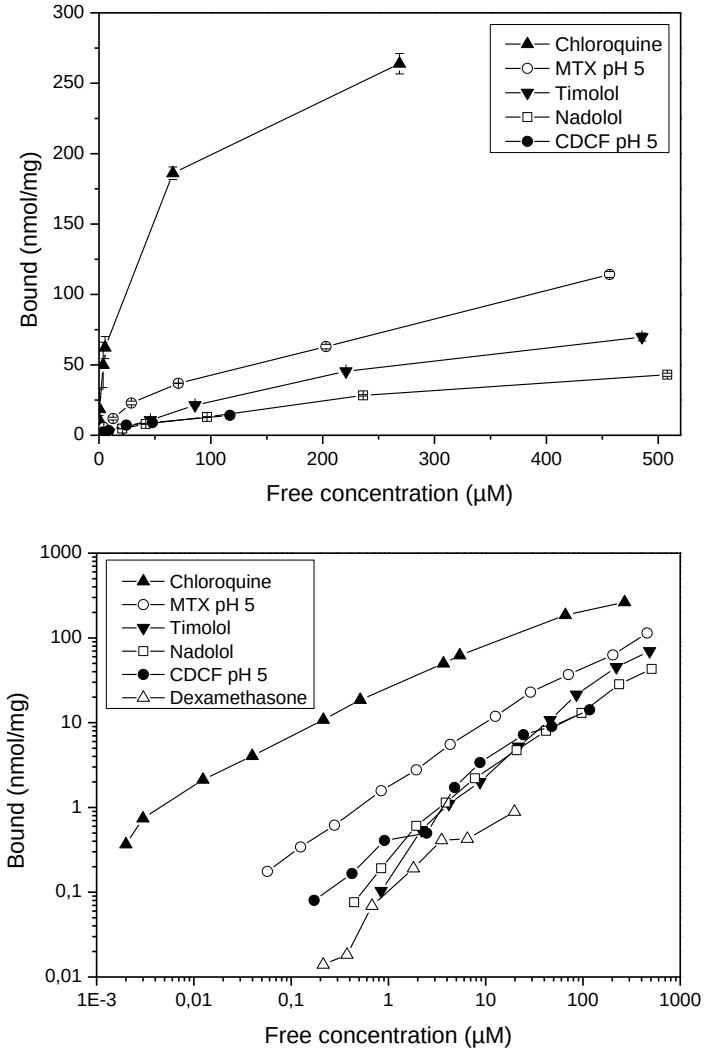

Figure 10. Binding of chloroquine, timolol and nadolol at pH 7.4, and methotrexate (MTX) and CDCF at pH 5 on a linear scale (upper graph) and on a log-log scale (lower graph). Dexamethasone is included in the log-log scale. Error bars show the standard deviation of replicate samples and are in most cases encompassed by the symbol. Error bars are not presented in the log-log scale.

Binding parameters B_{max} and K_d were calculated by nonlinear curve fitting to the Langmuir binding isotherm. Values for the parameters are presented in Table 10. With timolol, only experiments starting from the 1 µM concentration were considered, since there was a problem with the samples of the smallest concentrations, showing no binding or negative binding. With CDCF, only the experiments up to the starting concentration of 100 µM were considered, because of much larger standard deviations in the higher concentration experiments and a poor fit to the nonlinear binding isotherm. R^2 values were higher than 0.98 with both binding site models with all the compounds. For chloroquine and methotrexate (pH 5) the Scatchard plots showed upward concavity, which can indicate multiple classes of binding sites or negative cooperativity (Figure 11). For nadolol, timolol and CDCF (pH 5) the Scatchard plot was inconclusive. With methotrexate (pH 5) the one-site binding model seemed to give more reasonable values for the parameters. With chloroquine both models gave acceptable values. With dexamethasone no values for the binding parameters were obtained, most likely due to fluctuation in the results caused by the low amount of binding.

Table 10. Binding parameters of nadolol, timolol, and chloroquine at pH 7.4, and methotrexate (MTX) and CDCF at pH 5 with standard error in the parentheses.

One-site binding model					
	Nadolol	**Timolol**	**MTX pH 5**	**CDCF pH 5**	**Chloroquine**
B_{max} (nmol/mg)	80.0 (5.2)	138 (7)	140 (12)	19.6 (1.3)	271 (12)
K_d (µM)	437 (50)	471 (38)	204 (38)	47.5 (6.9)	21.2 (4.0)
Two-site binding model					
	Nadolol	**Timolol**	**MTX pH 5**	**CDCF pH 5**	**Chloroquine**
B_{max1} (nmol/mg)	1.65 (1.5)	61.5 ($9*10^7$)	39.2 (4.2)	11.9 (6.0)	42.7 (2.5)
K_d (µM)	5.80 (11)	471 ($7*10^7$)	33.1 (4.7)	26.7 (13)	0.785 (0.12)
B_{max2} (nmol/mg)	88.0 (8.9)	76.9 ($9*10^7$)	1 240 (2 200)	$2{,}3*10^7$ ($2*10^{14}$)	268 (3)
K_d (µM)	567 (130)	471 ($6*10^7$)	8 440 (17 000)	$6.2*10^8$ ($4*10^{15}$)	56.9 (2.8)

R^2 values > 0.98 in all cases.

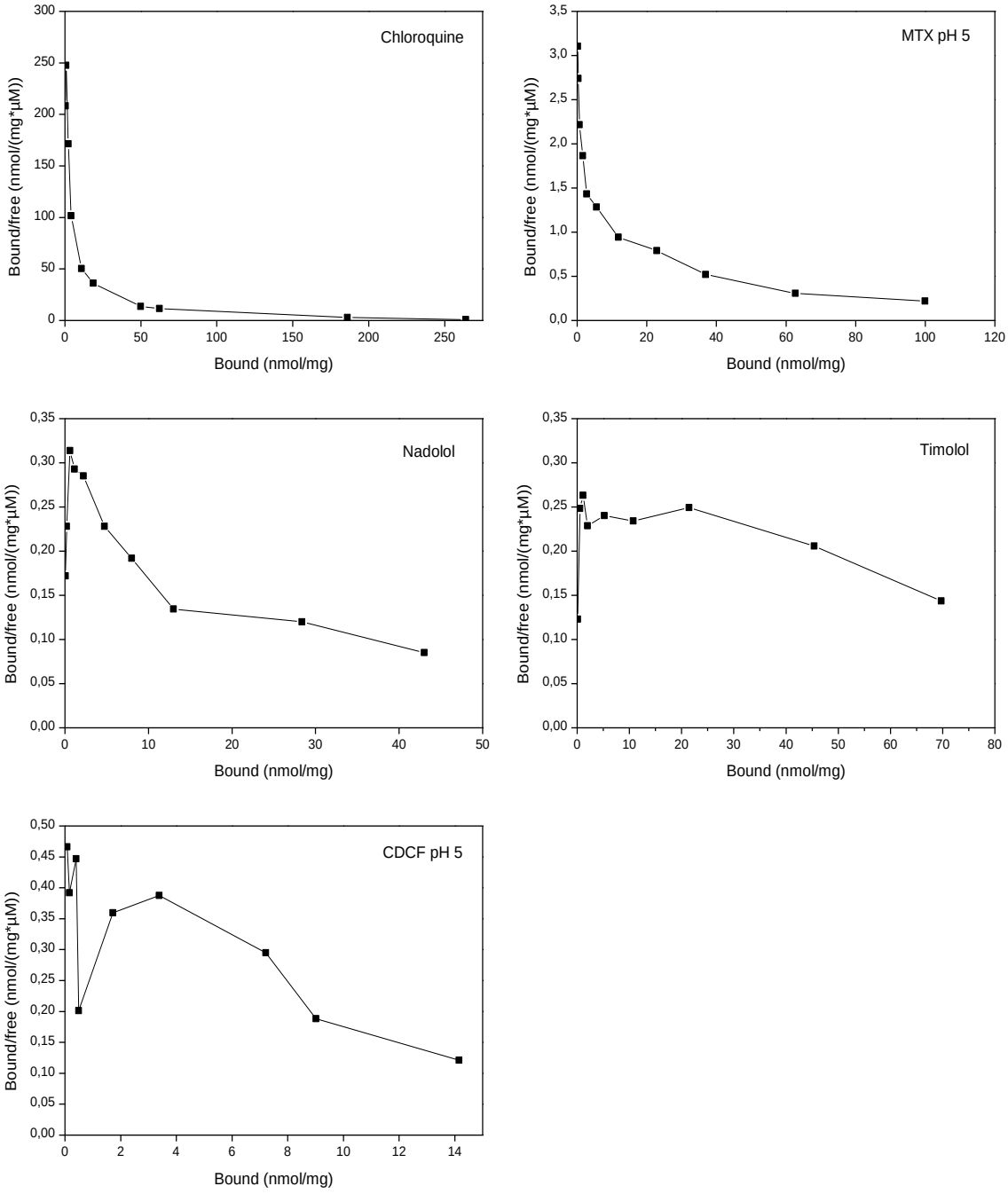

Figure 11. Scatchard plots for the binding of chloroquine, methotrexate (MTX) pH 5, nadolol, timolol and CDCF pH 5.

Hill coefficients are presented in Table 11. The coefficients were calculated with different values for the total amount of binding sites since the amount of binding sites is unknown. Timolol, nadolol and CDCF (pH 5) had Hill coefficients close to one indicating one class of binding sites and no cooperativity. Chloroquine and

methotrexate had values lower than one, also indicating one class of binding sites but with negative cooperativity in binding.

Table 11. Hill coefficients (n) calculated with different R_T values (*B_{max} value calculated from the equilibrium study results (one-site binding model)).

TIMOLOL		**NADOLOL**		**CHLOROQUINE**		**MTX pH 5**		**CDCF pH 5**	
R_T (nmol/mg)	n	R_T (nmol/mg)	n	R_T (nmol/mg)	n	R_T (nmol/mg)	n	R_T (nmol/mg)	n
138*	1,09	80*	0,96	271*	0,75	140*	0,80	19,6*	1,00
200	1,06	200	0,90	500	0,60	200	0,78	50	0,88
500	1,02	500	0,88	1000	0,57	500	0,76	100	0,86

7.2.3 Dissociation study

Chloroquine dissociation from melanin was measured at 0, 0.5, 1, 1.5, 2, 3.2, 5, 24, and 120 h (5 days) with starting concentrations 1 and 10 µM. Results are presented in Figure 12. The results did not give a good estimate of the dissociation rate constant. Fitting the nonlinear Equation 11 to the results gave R^2 values of 0.168 and 0.015 for the 1 and 10 µM concentrations, respectively. Fitting a linear equation to the results, R^2 values were 0.170 and 0.015.

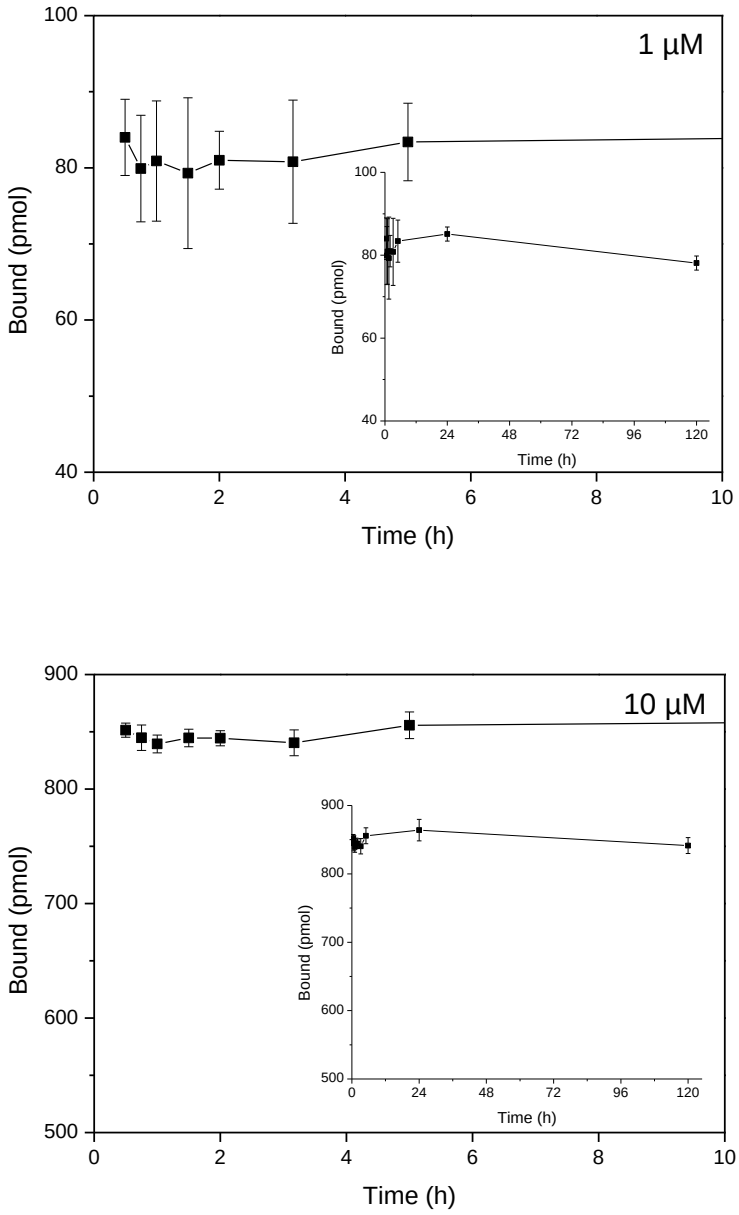

Figure 12. Dissociation of chloroquine from melanin with 1 µM (upper graph) and 10 µM (lower graph) starting concentrations. Error bars show the standard deviation of replicate samples.

Nadolol and timolol bound to such a small extent that after dilution of the system to initiate dissociation, the small concentration difference between time points could not be reliably analyzed by the mass spectrometric analysis used.

7.2.4 Association study

The association of nadolol to melanin was measured at time points 15, 30, 45, 60, 90, 120, 180 and 300 minutes at a melanin concentration of 1 mg/ml. The association was so fast that the association constant (k_{on}) was impossible to calculate in this study design (Figure 13).

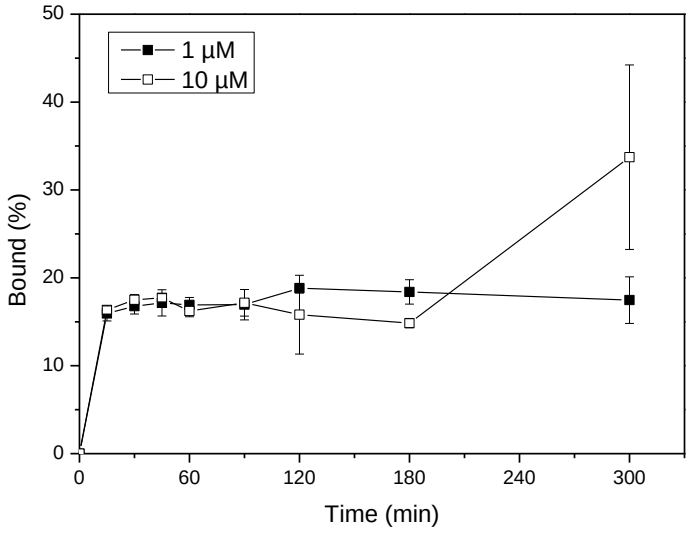

Figure 13. Association of nadolol to melanin with 1 µM and 10 µM concentrations. Error bars show the standard deviation of replicate samples.

7.3 Cell study

7.3.1 Cell culture

The cells were photographed 4, 8 and 14 days after the isolation and 1 day after seeding on the 48 well plate (Figure 14). Melanin granules were visible in all stages, although the amount of pigmentation seems to be less in later stages compared to day 4.

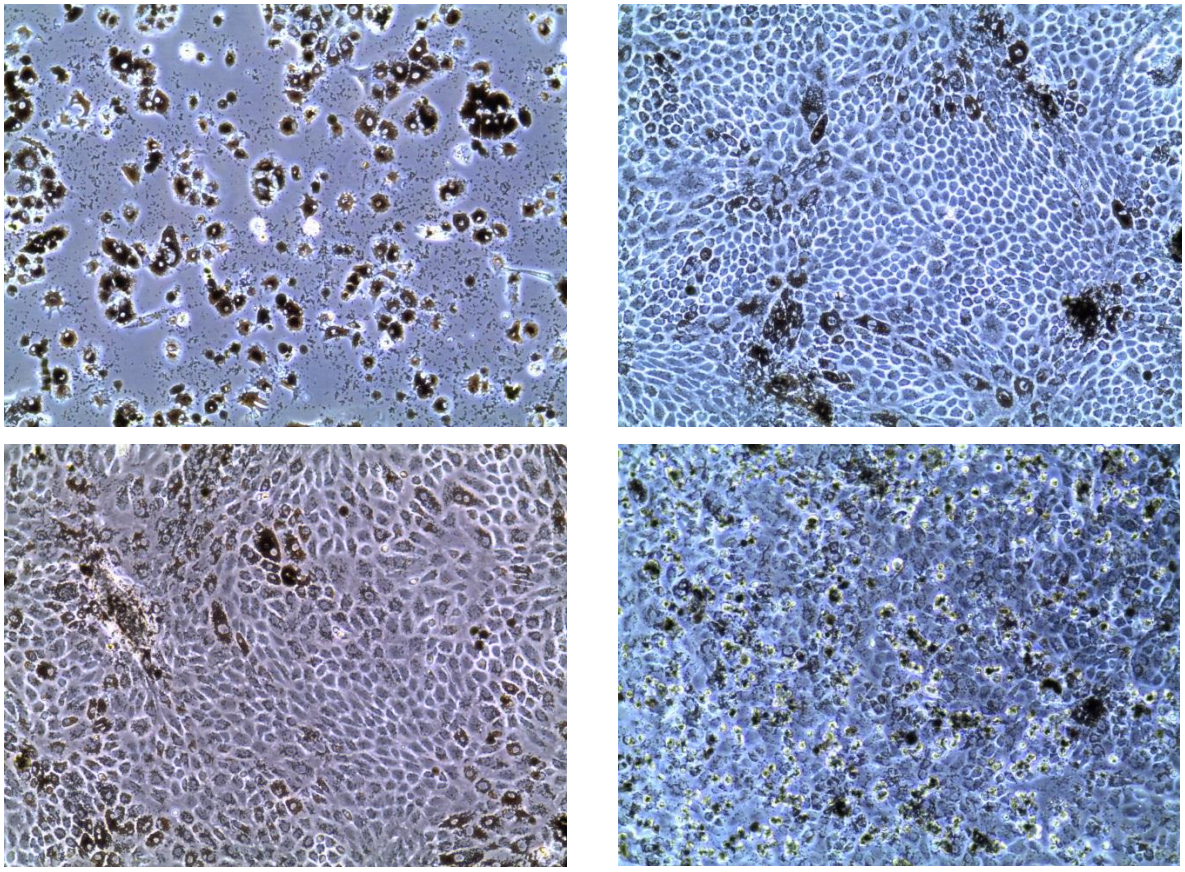

Figure 14. Microscope images of the second batch of pRPE cells (10x magnification) 4 (top left), 8 (top right) and 14 days (bottom left) after isolation and 1 day after seeding (bottom right) on the well plate.

7.3.2 Uptake study

The amount of chloroquine taken up by the cells was substantial. The starting concentrations were 0.34, 2.8 and 6.7 µM. An average of 91, 64, and 52 % of chloroquine was taken up by the cells at these starting concentrations, respectively. The results for the uptake study at time point 20 h are presented in APPENDIX 4.

The amount taken up by the cells was too small for reliable detection with dexamethasone, methotrexate, and CDCF from the samples taken from the medium. In the studies done with the inhibitor, the cells contained an average of 1.7 % of the total methotrexate in the wells and 1.4 % of the total CDCF. The cell study with nadolol was unsuccessful, since in the UPLC analysis there was another compound in the medium

that eluted at the same time as nadolol, and the amount of nadolol could not be quantitated. Due to limitation in the amount of RPE cells, nadolol was left out of subsequent experiments. In the case of timolol, it seemed that some of the drug was taken up by the cells, but the variation of the results was so large that the result was not statistically significant.

7.3.3 Elimination study

The results for the elimination of chloroquine from the cells are presented in Figure 15. The presence of probenecid had no significant effect on the results. When compared to the values of the uptake study, the amounts left inside the cells after the 48 hour elimination study were on average 84 % and 57 % of the amount taken up by the cells in the 0.34 and 2.4 µM studies, respectively.

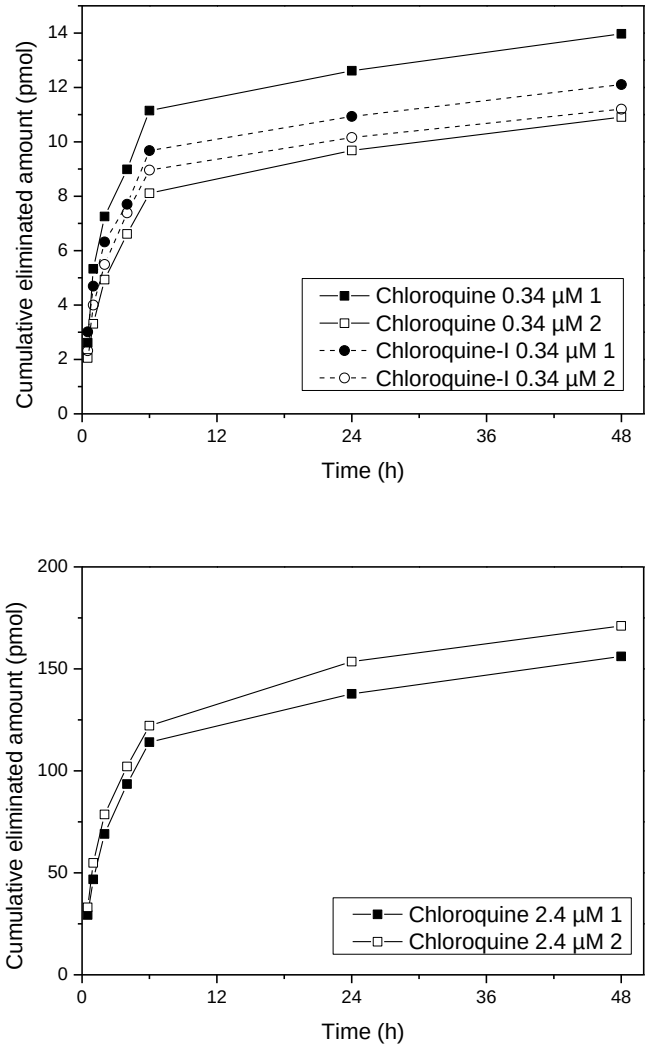

Figure 15. Elimination of chloroquine from pRPE cells (squares represent experiments without inhibitor, circles with inhibitor (probenecid = I), 1 and 2 are replicate wells).

The results for the elimination of timolol are presented in Figure 16. At both concentrations it is evident that the elimination occurred within the 48 h sampling period. After the 48 h period, the amount of timolol left in the cells was less than 2.5 % of the amount of timolol eliminated (between 0.5 and 48 h).

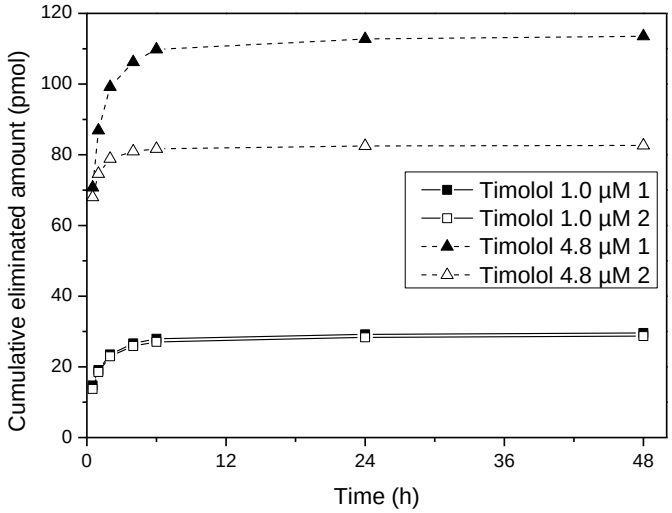

Figure 16. Elimination of timolol from pRPE cells at starting concentrations of 1 and 4.8 µM (1 and 2 are replicate wells).

The elimination study of CDCF was done in the presence of probenecid to maximize the probability of the amount inside the cells being measurable. The uptake in the presence of probenecid was rather scarce, since no uptake was detected in samples taken from the medium before initiating elimination. The elimination study, however, showed that CDCF was taken up by the cells, since elimination could be detected (Figure 17). After the 48 h study there was 22 % and 11 % of the amount of CDCF eliminated (between 0.5 and 48 h) left in the cells (replicate wells 1 and 2, respectively).

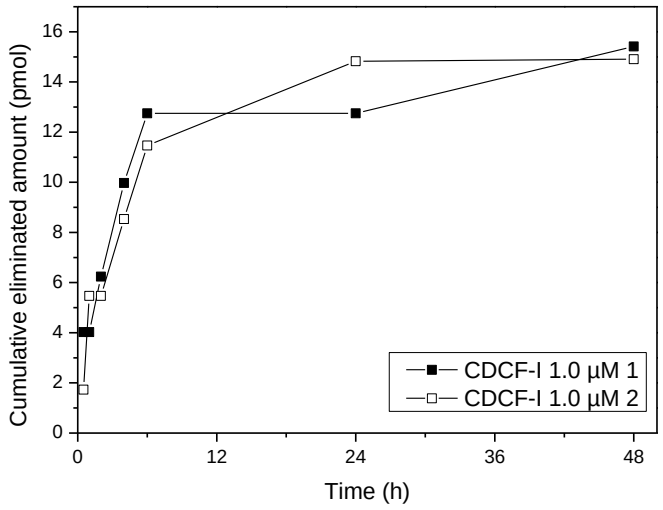

Figure 17. Elimination of CDCF from pRPE cells at a 1 µM starting concentration in the presence of probenecid (=I) (1 and 2 are replicate wells).

Methotrexate elimination was studied only in the presence of probenecid, since without the inhibitor the amount inside the cells was expected to be very small. The elimination was nonexistent, most likely because very little of methotrexate was taken up by the cells despite efflux inhibition.

7.4 Pharmacokinetic modeling

7.4.1 Modeling of melanin binding

Kinetic models for melanin binding with one or two binding site classes on melanin were constructed. Simplified models are presented in Figure 18, the whole STELLA® models with equations are found in APPENDIX 5.

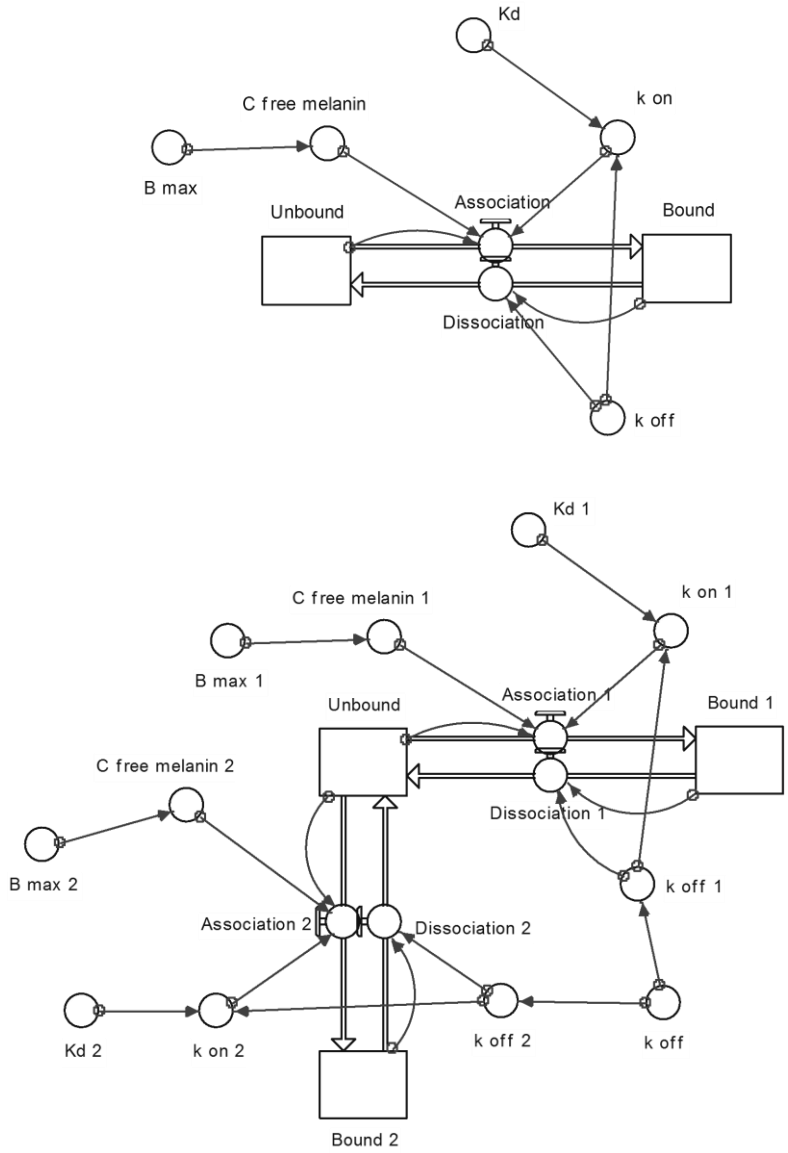

Figure 18. Pharmacokinetic models for melanin binding with one (upper model) and two (lower model) binding site classes.

Since no values for the dissociation rate constant were obtained from the binding study, the binding was simulated varying this constant. In the two-site binding model, the dissociation rate constants were determined to be the same for both sites, since a difference in these values changes the amount bound to melanin.

Dissociation rate constants (k_{off}) were determined so that equilibrium was reached within 5 hours (Table 12; APPENDIX 6). Changes in k_{off} did not affect the amount

bound to melanin, only the time to equilibrium. Binding curves for both one and two-site binding models are presented in Figure 19.

Table 12. Values for the dissociation rate constant (k_{off}).

k_{off} (h^{-1})	One binding site	Two binding sites
Timolol	0.6	-
Nadolol	0.7	0.5
Chloroquine	0.1	0.2
MTX (pH 5)	0.5	0.6
CDCF (pH 5)	0.5	-

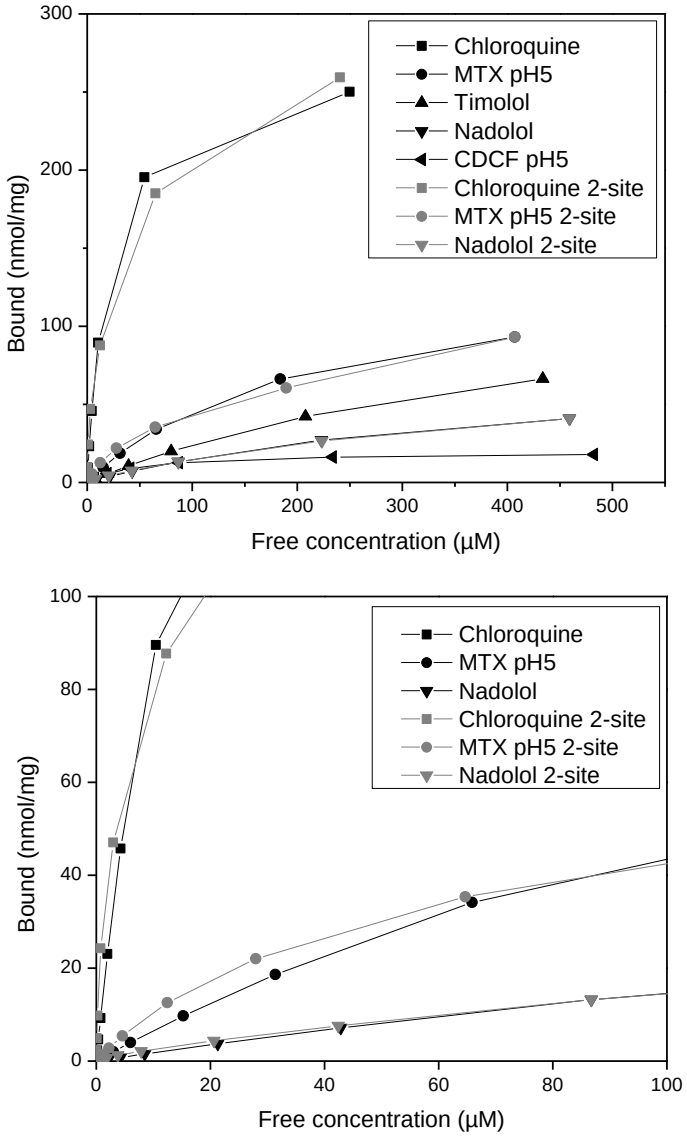

Figure 19. Modeled binding of chloroquine, timolol and nadolol at pH 7.4, and methotrexate (MTX) and CDCF at pH 5.

Dissociation was modeled with the same dissociation rate constants as equilibrium binding (Table 12). The equilibrium was allowed to reach equilibrium without sink conditions. The results are presented in Table 13.

Table 13. Modeled dissociation from melanin.

Compound	Binding model	Starting concentration (µM)	Fraction dissociated (%)	Unbound concentration (µM)*	Equilibrium time (h)**	k_{off} (h^{-1})
Nadolol	one-site	1	85	0,131	4	0,7
		10	85	1,29	4	0,7
	two-site	1	70	0,203	4	0,5
		10	73	1,56	4	0,5
Timolol	one-site	1	77	0,175	5	0,6
		10	77	1,73	5	0,6
Chloroquine	one-site	1	7	0,067	2	0,1
		10	7	0,690	2	0,1
	two-site	1	2	0,017	1	0,2
		10	2	0,200	1	0,2
MTX (pH 5)	one-site	1	59	0,241	4	0,5
		10	60	2,38	4	0,5
	two-site	1	43	0,245	3	0,6
		10	44	2,41	3	0,6
CDCF (pH 5)	one-site	1	71	0,205	5	0,5
		10	72	1,88	5	0,5

*Unbound concentration is entirely caused by dissociation, i.e. unbound concentration at t=0 is 0, the reported concentration is the equilibrium concentration

**Equilibrium assumed reached when 95 % of the total 'fraction dissociated' has dissociated and equilibrium time reported as the hour within which equilibrium is reached

7.4.2 Modeling of cell uptake with melanin binding

A cell uptake model with melanin binding was built for both one and two-site binding models (Figure 20; APPENDIX 5). The model was simulated with starting concentrations 1, 5 and 10 µM with the compounds that bound to melanin (Table 14; APPENDIX 7). The model was also simulated without melanin binding. In this case the concentrations inside and outside the cells are the same at equilibrium. The fraction inside the cells is therefore determined only by the ratio of the volumes of these compartments and is not related to the compound in question. Thus important

physicochemical properties, including logP and pK_a, are not taken into account in the model. The fraction of compound inside the cells with this model (without melanin) was 0.04 %.

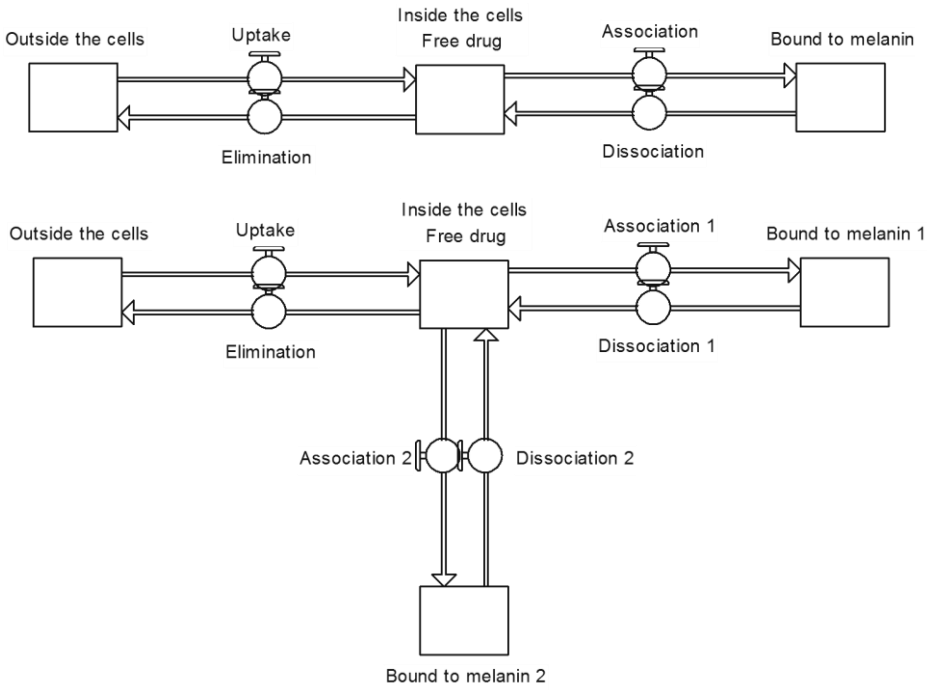

Figure 20. Pharmacokinetic cell models for one (upper model) and two (lower model) binding site classes on melanin.

Table 14. Modeled results of cell uptake.

Fraction inside the cells at equilibrium (%)			
Compound	**Starting concentration (µM)**	**One-site binding model**	**Two-site binding model**
Nadolol	1	5,2	11
	5	5,2	8,6
	10	5,1	7,3
Timolol	1	8,1	-
	5	8,1	-
	10	8,0	-
Chloroquine	1	79	-
	5	79	-
	10	78	-
MTX (pH 5)	1	17	28
	5	17	27
	10	17	25
CDCF (pH 5)	1	11	-
	5	10	-
	10	9,4	-

Fraction inside the cells without melanin: 0.04 % (all compounds)

When a STELLA model is complicated enough, a change in integration interval (DT) can change the results. When DT is set to be very small, the system will resemble a continuous system (Isee Systems 2010). This is desired in pharmacokinetic modeling. However, the smaller the DT, the shorter is the possible length of a simulation. For chloroquine, no results were obtained with the model with two binding sites since it was not possible to set the integration interval (DT) low enough to assure steady results and still be able to reach equilibrium.

8 DISCUSSION

8.1 Characterization of melanin granules

Melanin is known to aggregate in solution over time (Pitkänen et al. 2007). The microscopical examination of the particle size showed that melanin formed some larger aggregates in suspension during the 20 hour incubation. Aggregation may affect the

results of binding studies, since the size of the surface area affects the number of binding sites available and thus the bound amount (Pitkänen et al. 2007). However, aggregation was most significant after 20 hours of incubation, compared to the earlier time point at 5 hours. Since melanin binding is rather fast, the compound has time to bind before aggregation is considerable. This reduces the effect of aggregation on the results.

There is reasonably little information in literature about the zeta potential of melanin. Mani et al. (2001) measured the zeta potential of synthetic melanin getting zeta potential values of -12 mV (pH 5) and -45 mV (pH 7). These results resemble the measured zeta potential of melanin in this study (on average -16 mV (pH 5) and -25 mV (pH 7.4)) although there is a significant difference in the near neutral pH results. Differences between these studies can however be expected, since synthetic and natural melanins are being compared.

A difference in zeta potential at different pH values can be expected to affect melanin binding, especially with charged compounds, since it changes the strength of ionic interactions. Mani et al. (2001) showed also that proteins bound to melanin change the zeta potential of the complex to the more positive direction. Since melanin is always bound to proteins *in vivo*, this is expected to affect melanin-drug binding. However, Larsson and Tjälve (1979) have shown that protein moieties bound to natural melanin do not affect the characteristics of this binding. More results are needed on this matter.

8.2 Binding study

8.2.1 Experimental conditions

The binding study was done with melanin from porcine RPE-choroid. There are known differences between results achieved using melanin from different sources (Koeberle et al. 2003; Pitkänen et al. 2007). This affects the comparison of results obtained with melanin from different sources and the prediction of drug binding in human RPE-choroid. Since the availability of human ocular melanin is limited, melanin binding

studies are mostly done with isolated bovine, *Sepia* or porcine melanin or with synthetic melanin (Potts 1964; Shimada et al. 1976; Koeberle et al. 2003; Pitkänen et al. 2007; Pescina et al. 2012). Using these results as such in prediction of human ocular melanin binding may cause some errors. There is little information in literature about differences in the structure of melanin from different sources and different isolation techniques. Also the composition of the experimental environment (suspension) may affect the results, since e.g. metal ions in the suspension are shown to affect the magnitude of melanin binding of other compounds by binding to melanin themselves (Wrzesniok et al. 2012). More comparative studies with different melanin sources should be performed and methods developed to ease the comparison of results from different binding studies. For example, the binding should be normalized to the surface area of the melanin granules, since surface area affects the amount bound (Pitkänen et al. 2007). In this study, the surface area of melanin granules was not analyzed.

The binding studies, other than with methotrexate and CDCF, were done only at physiological pH 7.4. The pH of ocular melanosomes is expected to be acidic, thus pH is a factor in melanin binding. The results at pH 5 and 7.4 were very different for methotrexate and CDCF. At pH 7.4 methotrexate had no binding, but at pH 5 the binding reached 75 % at the smallest concentrations. Methotrexate is a dicarboxylic acid with pK_a values of 4.1 and 3.4. It is virtually entirely in its anionic form at pH 7.4. At pH 5, however, some of the molecule is in the neutral form. Thus it is expected that the binding of methotrexate changes with the pH, as the neutral form binds more easily with melanin, a polyanionic polymer. The pH also affects the structure of melanin and its binding sites. Since melanin is polyanionic, the lower the pH, the more binding sites are expected to be in a neutral form. The zeta potential measurement showed that there was a difference between melanin charges at the two pH values used; the potential being more negative in the higher pH. This gives evidence of the changing electrostatic nature of the melanin in these pH values. The less negative potential of melanin would further ease the binding of methotrexate at pH 5. CDCF, an acid with a pK_a of 5.1, showed similar behavior at these two pH values. While no binding occurred at pH 7.4, there was ca. 30 % binding at the smallest concentrations at pH 5.

Melanin binding *in vivo* is more complex than the mere interaction between melanin and the drug. The pH varies in different parts outside and inside the cell. There can be metabolism, binding to other cellular components or other kinetic factors (e.g. extracellular distribution of the compound) (Larsson 1993). The melanin inside melanosomes is covered by a lipid membrane that the drug has to penetrate before it can bind. As the pH outside the melanosome is closer to 7.4, the pH difference especially affects the permeation of acidic drugs with pK_a values lower than 7.4. The binding study done at pH 5 would most likely give results that are not reflected in *in vivo* or cell studies, since the drug does not have similar access to the actual melanin. At pH 5 however the binding study would give a more accurate result of the intrinsic interaction between the drug and melanin. In the cell study, the amounts of methotrexate and CDCF inside the cells were comparable to dexamethasone, a low binding very weak acid (pK_a 11.4) that is neutral at both pH values and much less than with chloroquine, a strongly binding base (pK_a 8.1 and 10.4), that is cationic in both pH values. Thus it would seem that the binding result of melanin at pH 5 is not reflected in this cell study. However, a negative control was missing, since cells without melanin were not used as comparison. This makes it hard to draw reliable conclusions of the effect melanin binding has on the cell uptake and cellular kinetics of methotrexate and CDCF.

The decision about the pH in which melanin binding studies are performed should be made based on the aims of the study. In situations where the result is expected to only depict the intrinsic interaction with the drug and melanin, a lower pH would be more accurate. These results could be beneficial in a pharmacokinetic model where the lipid membrane of melanosomes is taken into account separately. Based on this study, it would seem that the results at pH 7.4 are in relation with the results of the cell study, and thus depict the *in vivo* situation better. However, the material of this study is limited and literature on this matter is sparse; therefore more studies on the subject should be performed.

8.2.2 Results of the binding study

The results of the equilibrium binding study were fitted to a Langmuir binding isotherm (Equations 2 and 10). Usually either one or two-site binding model is selected based on the Scatchard plot (Buszman and Rozanska 2003; Pitkänen et al. 2007). In other studies, however, a decision to use the one-site binding model is made based on the principle of using the simplest model that fits (Koeberle et al. 2003; Pescina et al. 2012). Melanin is a random polymer and different modes of interaction are present in the binding process (Larsson et al. 1993; Koeberle et al. 2003). Therefore it is difficult to differentiate between separate binding site classes. Most melanin binding studies only present results of nonlinear curve fitting to one of the two binding models and do not compare the differences between the results given by these models. In this study, the results were fitted to both models and compared. There were no distinct differences between the goodness of fits, but p-values for the binding parameters varied greatly. Scatchard plots were indicating two classes of binding sites for chloroquine and methotrexate (pH 5). This shape of a Scatchard plot can also indicate binding with negative cooperativity (Invitrogen 2006). Thus the binding was also analyzed with the Hill plot. With the other compounds Scatchard plots were inconclusive. In most cases the Hill plot gave values close to one, suggesting towards the one-site binding model. With chloroquine and methotrexate, however, the Hill coefficient hinted of negative cooperativity in binding instead of two separate binding site classes. This kind of binding deviates from Clark's theory of binding, where there should be one ligand independently interacting with one receptor with no cooperative binding (Invitrogen 2006). Deviations from Clark's theory make it harder to evaluate binding parameters with equations like the Langmuir binding isotherm. The Hill equation is mostly used in receptor binding, which is very specific in nature (Invitrogen 2006). Melanin binding, at least in this study, would seem to resemble nonspecific binding with low affinity, high capacity binding sites. Thus it is difficult to assess the accuracy of the Hill equation in melanin binding, since melanin binding is somewhat different compared to receptor binding.

Considering the pharmacokinetic model, the nature of melanin binding (i.e. one-site or two-site binding model) is not significantly relevant. The amounts and fractions bound at different concentrations are more important. This was modeled with the kinetic model constructed of melanin binding, which is discussed later.

As expected based on literature, chloroquine had the strongest binding to melanin of the studied compounds. Timolol and nadolol also bound to melanin, as expected (Aula et al. 1988; Kadam and Kompella 2010). Literature of methotrexate binding was ambivalent, with studies reporting either binding or no binding at neutral pH (Tsuchiya et al. 1987; Wilczok et al. 1990). At pH 5, binding was expected (Tsuchiya et al. 1987). Dexamethasone, as expected, had low binding (European Medicines Agency 2010). No results were found on the binding of CDCF, but a closely resembling molecule 6-carboxyfluorescein did not bind at pH 7.4 (Pitkänen et al. 2007). From this point of view, the binding study results were in line with literature.

Variation in the results for the parameters K_d and B_{max} between different studies is somewhat large (Table 15, timolol). This may be a result of different melanin isolation techniques or melanin sources, different assay conditions or even different analytic methods (Tsuchiya et al. 1987). Only a few studies with porcine ocular melanin were found (Buszman and Rozanska 2003; Buszman et al. 2008; Pescina et al. 2012). These studies did not contain any of the compounds of this study. Comparing the values of these parameters to other literature is therefore challenging. Results from literature have been collected to Table 15. For the compounds in this study, all literature results were calculated with the one-site binding model.

Table 15. Literature values for binding parameters (B_{max}, K_d) and experimental values of this study analyzed with the one-site binding model.

Compound	Parameters	This study	Literature	
Timolol	B_{max} (nmol/mg)	138	6,98[1]	233[2]
	K_d (µM)	471	6,00	45,1
	Melanin	porcine	Sepia	synthetic
Nadolol	B_{max} (nmol/mg)	80	8,91[1]	
	K_d (µM)	437	6,20	
	Melanin	porcine	*Sepia*	
Chloroquine	B_{max} (nmol/mg)	271	1130[2]	
	K_d (µM)	21,2	2,35	
	Melanin	porcine	synthetic	
Methotrexate (pH 5)	B_{max} (nmol/mg)	140	60,0[3]	63,0[3]
	K_d (µM)	204	42,7	92,6
	Melanin	porcine	synthetic	B16 melanoma
CDCF (pH 5)	B_{max} (nmol/mg)	19,6	-	
	K_d (µM)	47,5	-	
	Melanin	porcine	-	

Dexamethasone: no results for binding parameters were obtained in this study or found from literature
[1] (Kadam and Kompella 2010)
[2] (Ono and Tanaka 2003)
[3] (Wilczok et al. 1990) literature results obtained at pH 7

From the dissociation study, no useful results were obtained. During the 5 day study frame, chloroquine did not seem to dissociate significantly from melanin. Some dissociation could be detected by the last time point (5 days). The dissociation of chloroquine from melanin is known to be very slow as it can be found in pigmented tissues even a year after a single intravenous injection (Larsson 1993). Thus the time frame for the study may have been too small to detect dissociation. The stability of the study system, however, might be questionable during a longer study, since melanin is known to aggregate in solution over time as shown in this study and others (Pitkänen et al. 2007). When designing a study to determine the dissociation rate constant, this should be taken into account with compounds that dissociate slowly.

The dissociation of compounds that have low binding (less than 25 %) should be measured with analytic methods sensitive enough to distinguish the small concentration differences that the dissociation of the compound causes. In this study, the mass spectrometric method used was not sensitive enough to make this distinction. In

dissociation studies, mostly radiolabelled compounds have been used (Aula et al. 1988; Aula et al. 1989).

There is very little information available of the dissociation of drugs from melanin. Dissociation has mostly been studied *in vivo* by using autoradiography to assess the prevalence of the drug in pigmented tissue after dosing has ended (Bathory et al. 1990; Trope et al. 1994). This information would be useful for the pharmacokinetic model of binding.

In the association study the association of nadolol to melanin seemed to be so fast that it was hard to measure accurately enough to calculate the association rate constant (k_{on}). In the kinetic studies performed before the association study, the association seemed to be somewhat slower, the equilibrium being reached between 3-5 hours. However, defining an exact value for k_{on} is not relevant to the pharmacokinetic binding model, since association is a faster process than dissociation.

A correlation between the physicochemical properties of the compounds studied and their binding to melanin could be noticed. All basic compounds (timolol, nadolol and chloroquine) bound to melanin at pH 7.4. The acidic compounds had either no binding (methotrexate and CDCF) or low binding (dexamethasone) at pH 7.4. Chloroquine, the most lipophilic of the compounds, had the highest binding and the most hydrophilic compounds had the lowest binding (i.e. no binding) at pH 7.4. Since the amount of compounds studied was small, quantitative structure property relationships cannot be made.

8.3 Cell study

In the cell uptake study only chloroquine seemed to accumulate into the cells. Chloroquine is known to strongly bind to melanin and to remain in pigmented tissues for long periods of time (Larsson 1993). However, chloroquine is also known to accumulate into lysosomes (Schraermeyer 1999). To obtain reliable parameters of the

effect melanin binding has on the pharmacokinetics of chloroquine, it would be important to have control cells that do not contain melanin.

The elimination of chloroquine seemed to be nonlinear. However, an equilibrium state was most likely reached, since the amount of compound eliminated from the cells at each time point did not seem to increase although the time between each sample (i.e. time point) increased as the study went on. More thorough sink conditions, i.e. more frequent medium changes, in the cell elimination studies would have been necessary.

Timolol, another melanin binding drug in the cell study, did not seem to be retained inside the cells. The elimination took place within the 48 hour sampling period. However, timolol has been shown to accumulate into pigmented ocular tissues, especially after prolonged exposure (Salminen and Urtti 1984; Trope et al. 1994). Here as well, a negative control of cells without melanin would have helped to evaluate the effect melanin had on the elimination phase.

The efflux inhibitor probenecid did not seem to have an effect on the elimination of efflux substrates chloroquine, CDCF or methotrexate. The effect on uptake of these compounds was left unclear, since there was a problem with the ‘inside the cell’ samples in the inhibitor studies. The samples from inside the cells did not have the amount of compound expected based on medium samples and mass balance. This may be due to a failure in the lysing of the cells. The compound may have been left bound to cell components, among others melanin. Human retinal pigment epithelial cell lines are expected to express efflux transporters MRP1, MRP4 and MRP5 (Mannermaa et al. 2009). Porcine RPE has been shown to express P-glycoprotein (P-gp) and MRP1 (Steuer et al. 2005). Chloroquine and methotrexate are MRP1 and P-gp substrates and CDCF an MRP5 substrate. Since efflux expression in the pRPE cells used was not studied, the repertoire of efflux transporter expression in these cells is not known with certainty. Thus drawing reliable conclusions of the effect of efflux based on this study is difficult. Also, a more sensitive analytic method, possibly radiolabeling, should be used when assessing the small differences in drug uptake caused by the inhibitor.

Some improvements to the cell study design could have been made. Some samples were left unanalyzed, since medium samples showed only minor drug uptake. Without significant melanin binding, as with chloroquine, the amount taken up by the cells was so low, it could not be reliably detected in the medium samples, but could have been detected in the cell samples. There may have been some noticeable differences in the amounts taken up by the cells left undetected. This, combined with the problems with cell samples in the inhibitor and elimination studies, made it difficult to obtain parameters for the pharmacokinetic model from this cell study. Also, considering the pharmacokinetic model, it would have been beneficial to have a negative control, i.e. similar cells without melanin, in the cell studies. This would ease the comparison between results from the cell studies and results from melanin binding studies. Knowing the effect melanin binding has on cellular pharmacokinetics helps to create a pharmacokinetic model of the effect on ocular pharmacokinetics and possibly enables the use of a model with parameters from melanin binding studies alone.

8.4 Pharmacokinetic modeling

8.4.1 Melanin binding model

Melanin binding was modeled with both the one and two-site binding models. In the one-site binding model the variation of k_{off} did not cause any difference in the amount bound to melanin, but changed the time till equilibrium was reached. In the two-site binding model, a difference in k_{off} values for the two binding sites caused differences in the amount bound to melanin. Thus the values were determined to be the same for the separate binding sites. The reality of this situation remains unknown, since there is very little information in literature of melanin dissociation rate constants. The few studies found depict melanin binding with the two-site binding model but give one value for the dissociation constant (Aula et al. 1988; Aula et al. 1989). Thus at this point it is informed to use only one value for this constant.

With chloroquine and methotrexate (pH 5) the Scatchard plot could be considered an indication of two binding site classes. Therefore, it can be debated whether the one or

two-site binding model should be used in the model. When equilibrium binding was simulated with both models, it was evident that the two-site binding model gave values closer to experimental values, especially with the smaller test concentrations. At the higher concentrations the difference between the two models was smaller or nonexistent. Since the therapeutic concentrations of ocular drugs are usually not higher than 10 µM, it would be important to concentrate on depicting the smaller concentrations better (Ferencz et al. 1999; Chang-Lin et al. 2011). Thus it might be unnecessary to perform binding experiments with concentrations as high as was done in this study. The binding results of the higher concentrations seemed to distort the binding equation so that it gave less accurate values for the smaller, and from a therapeutic viewpoint more important, concentrations.

The modeled dissociation from melanin was rather fast. The modeled results, especially in the case of the equilibrium time, may not depict the real situation, since no values for k_{off} were obtained from the binding study. Chloroquine, which is expected to dissociate slowly, seemed to dissociate quite rapidly since a somewhat large k_{off} value was used. Chloroquine also reached equilibrium with less than one tenth of the bound amount dissociated, therefore the reality of the dissociation process *in vivo*, where the conditions resemble sink conditions, was not depicted. Since there is very little information of dissociation rate in literature, the reality of dissociation from melanin *in vitro* remains unknown.

Pharmacokinetic modeling can help in the design of melanin binding and cell studies by giving an idea of the expected events in the study. Study concentrations and needed incubation times are among the parameters that can be designed more easily with the help of kinetic simulations. In the dissociation study with chloroquine, the study time frame was thought to be too small (5 days). However, according to the kinetic model, this was most likely not the case, since the dissociating chloroquine quickly reaches equilibrium where according to the one-site binding model 7 % and the two-site binding model 2 % of the bound amount has dissociated. This dissociation causes a concentration difference of 67 or 17 nM (1 µM starting concentration) or 690 or 200 nM (10 µM). The higher concentration differences could have been detected, but the

standard deviations in the dissociation study were so high, that getting statistically significant results would have been hard. This shows that creating sink conditions in *in vitro* dissociation studies would be important, especially with high binding compounds. Without pharmacokinetic modeling, this conclusion could have been left undiscovered in this study.

8.4.2 Cell model

The constructed cell model is a very simple model that does not take into account many factors happening inside the cells. Among these are the intracellular distribution of a drug, efflux transporters, and melanin being inside lipid membrane covered melanosomes. For example, chloroquine is known to accumulate into lysosomes, which would significantly affect the cellular kinetics of the drug (Schraermeyer et al. 1999). More cell studies should be performed to be able to take these factors into account in the model.

Cell uptake results of chloroquine were similar to the simulated results. In the model, 78-79 % of chloroquine was taken up by the cells with concentrations between 0.3 and 10 µM. The fraction inside the cells being virtually the same with all the concentrations means that the differences in the free concentration inside the cells with the different starting concentrations is not large enough to cause a difference in melanin binding. The amount of melanin and thus its binding capacity in the model is very large, therefore a very large concentration of the drug is needed to cause a change in the fraction bound to melanin. In the *in vitro* cell study the amounts of chloroquine taken up by the cells were 91 % (0.34 µM), 64 % (2.8 µM) and 52 % (6.7 µM) of the starting amount. Since a negative control was missing, it is difficult to evaluate the fraction of these amounts that can be accounted to melanin binding. It can be assumed however, that a part of the differences in *in vitro* and simulated results are caused by intracellular distribution of chloroquine not taken into account in the model. Differences in these results can also be caused by differences in the amount of melanin inside the cells. The amount of melanin in the model was evaluated from the amount in human RPE. In the *in vitro* cell study

porcine RPE cells were used, and the cells most likely did not contain as much melanin because of cell division with no melanogenesis.

In the simulated model, 5 % of nadolol and 8 % of timolol were taken up by the cells. In the *in vitro* cell study, nadolol was left out of cell experiments, but timolol was studied and seemed to be taken up by the cells. The amount of timolol inside the cells was unclear, but some uptake could be detected in the medium samples of the uptake study and also in the elimination study. It is possible that the *in vitro* result would be in line with the modeled result but again reliable conclusions cannot be drawn because of incomplete *in vitro* results and lack of negative control.

When using the binding parameters at pH 5, the simulated amounts of methotrexate and CDCF taken up by the cells were 17 and 10 %, respectively. In the *in vitro* cell studies, no uptake was detected with methotrexate and the uptake of CDCF was very low. Thus it seems the simulated results do not correlate with the *in vitro* results. This indicates that the binding study results obtained at the lower pH do not depict the situation with cells and thus the *in vivo* situation. This is most likely due to the limited access of these hydrophilic compounds to the cells.

Elimination from the cells was not simulated, since no useful dissociation or elimination parameters were obtained from the *in vitro* studies. The elimination in the model would have therefore been a relatively fictitious situation, since in the model the rate of elimination was described only by the dissociation rate constant (k_{off}) and cell membrane permeability (P_{app}).

In the cell model, the only parameters determining the amount of drug inside the cells were the maximum binding capacity (B_{max}) and equilibrium dissociation constant (K_d) obtained from the binding study. This model is too simple for accurate evaluation of cellular pharmacokinetics, but gives an idea of the effect melanin binding can have. When constructing a more accurate model of the effect of melanin binding, the constants above should have an important role, if the desired result is be to be able to evaluate cellular pharmacokinetics based on binding study results alone. Considering

the large differences of results in different binding studies, it is important that the parameters are obtained from studies with same experimental conditions (melanin source, isolation technique) or are somehow normalized to correlate better with each other.

8.5 Future perspectives

Although melanin binding has been studied widely, there are factors left to be determined in the field, especially considering pharmacokinetic modeling. As mentioned earlier, there is rather little information in literature about drug dissociation from melanin. It would be important to study dissociation rate constants more widely, and determine how much of the retention of drugs inside melanocytes can be accounted for by retention in melanin. Considering pharmacokinetic modeling, the dissociation rate constant would also be important to know, as well as the correlation between calculated ($k_{on}=k_{off}/K_d$) and measured association rate constants (k_{on}).

Since differences in experimental conditions, including melanin isolation methods and different sources of melanin, can affect the results of melanin binding studies, it would be relevant to do comparative studies to elucidate the factors causing these differences. A way to compare results obtained in different conditions could be relating binding parameters to the surface area of melanin (Pitkänen et al. 2007). There is rather little information of this in literature, therefore more experiments should be conducted on the matter.

More studies of melanin binding in cells are needed. There are very few results on quantitative uptake of drugs into melanin containing cells. Studying the binding in cell cultures would help to relate binding study results done with melanin to the *in vivo* situation.

Melanin binding has not been included in detail into pharmacokinetic models of the eye and ocular drug delivery (Ranta and Urtti 2006; Ranta et al. 2010). The model built in this study was a very simple cell level model and did not take into account many factors

relevant to cellular pharmacokinetics. More information is needed for a more comprehensive model and for the inclusion of a cell model to a wider drug delivery model. Relating the physicochemical properties of a drug to melanin binding more accurately than already done would help in the modeling. In general, more comprehensive models of melanin binding are needed, to ease the evaluation of melanin binding in drug discovery and possibly take advantage of the binding in drug design.

9 CONCLUSIONS

Melanin binding of drugs is an important matter to consider in drug delivery. The binding is known to affect the pharmacokinetics of drugs especially in the eye because of densely pigmented tissues, like the RPE and iris. The physicochemical properties of a drug affect its melanin binding, as shown in this study and others. Although melanin binding has been studied numerously *in vivo* and *in vitro*, no comprehensive quantitative or pharmacokinetic models of melanin binding have been constructed. The results of *in vitro* binding studies and cell studies can be used in a pharmacokinetic compartmental model, however much more results are needed to construct a comprehensive model. A method for a better comparability of binding study results from different studies obtained with different sources of melanin and different study conditions should be created and variation causing factors should be determined. This would ease the use of these results in modeling as well as the comparison of melanin binding of different compounds. The kind of cellular level pharmacokinetic model constructed in this study, although a very simple model, has not been published before. To further improve this model, it should be modified to take into account many important factors affecting cell level kinetics.

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APPENDIX 1. Formulations of PBS and citrate buffer.

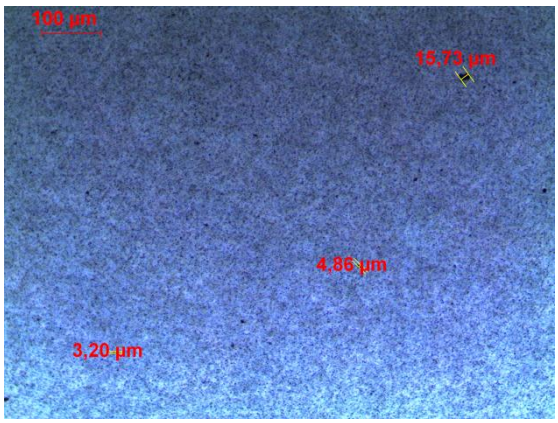
PBS pH 7.4

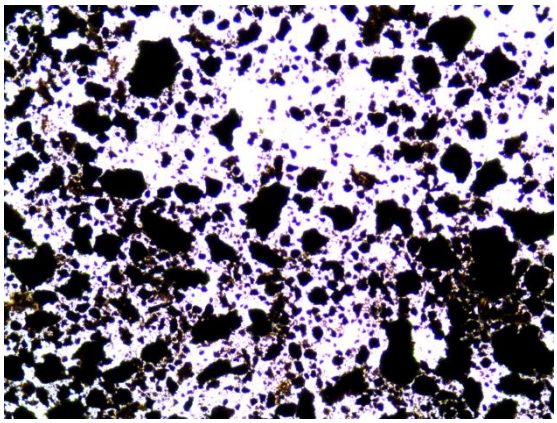
Per 100 ml of PBS:
21.0 mg of Potassium Phosphate monobasic (KH_2PO_4)
0.900 g of Sodium Chloride (NaCl)
72.6 mg of Sodium Phosphate dibasic (Na_2HPO_4-$7H_2O$)

Citrate buffer pH 5

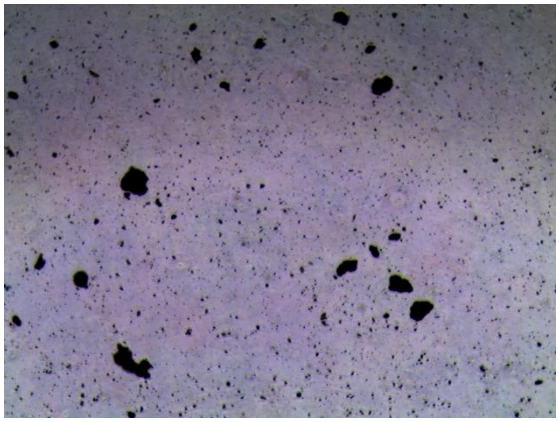
Per 100 ml:
20.5 ml of 0.1 M solution of citric acid (i.e. 21.0 g in 1 L)
29.5 ml of 0.1 M solution of sodium citrate (29.4 g $C_6H_5O_7Na_3$-$2H_2O$ in 1 L)

APPENDIX 2. Microscope images of melanin granules in suspension.





Light microscope images of a freshly sonicated 1 mg/ml melanin suspension (left) and a 20 h incubated melanin suspension (right). The scale is the same in both images.



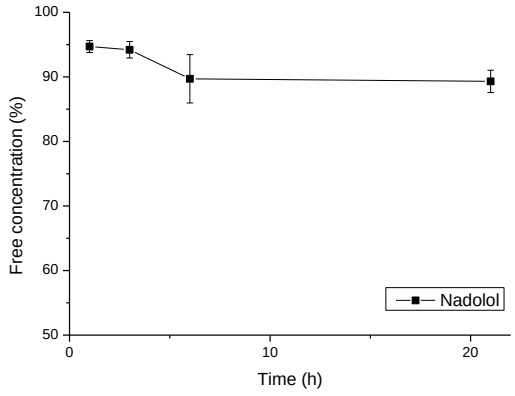
5 h incubated 0.2 mg/ml melanin suspension. The scale is the same as in the upper images.

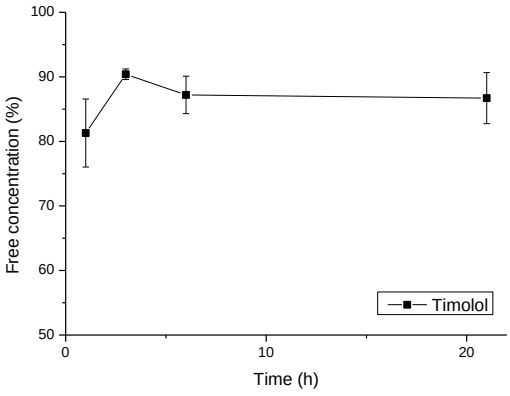
APPENDIX 3. Results of the kinetic study.

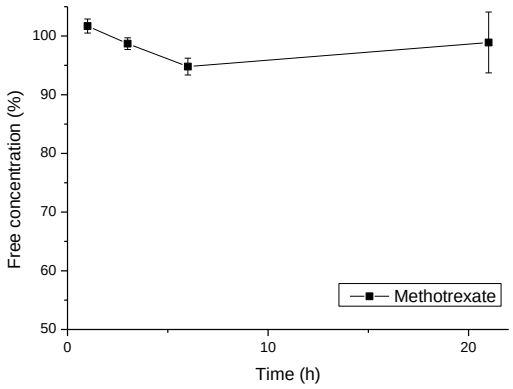
Kinetic studies were performed to determine the time it takes to reach equilibrium. Melanin concentrations used were 0.5 mg/ml and 1 mg/ml. The concentrations of the drugs were 0.1 µM for CDCF and 1 µM for the other drugs.

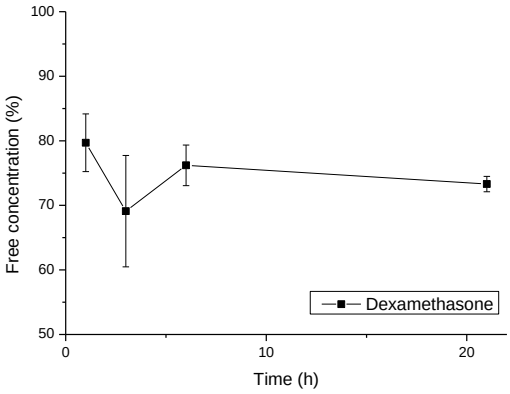
Time to equilibrium

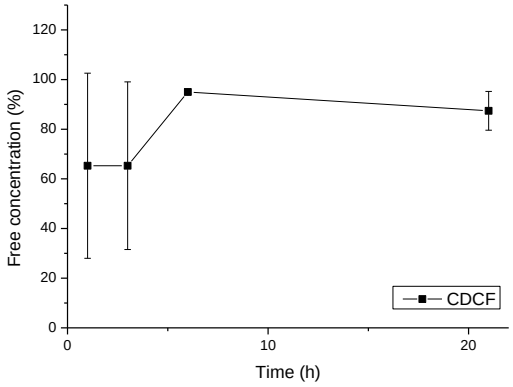
Melanin concentration 0.5 mg/ml



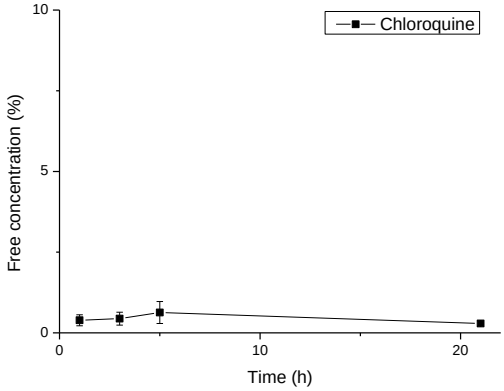


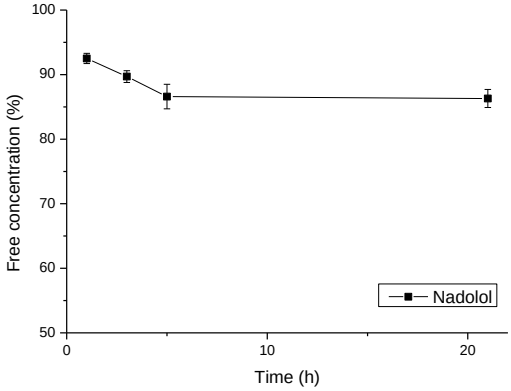






Melanin concentration 1 mg/ml





APPENDIX 4. Cell uptake of chloroquine.

Chloroquine uptake study results after 20 h incubation and the mass balance for each well.

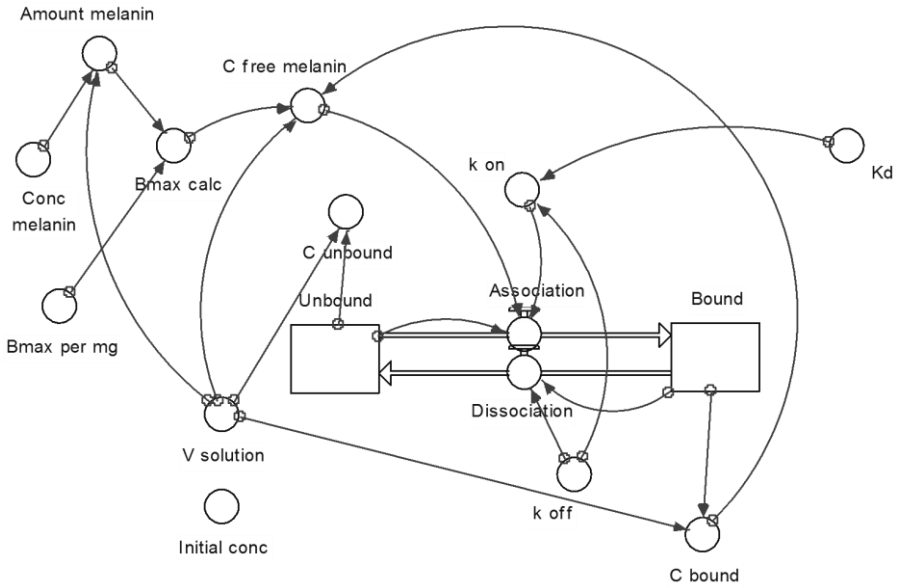
Wells	1µM well 1	1 µM well 2	5 µM well 1	5 µM well 2	10 µM well 1	10 µM well 2
Starting concentration (µM)	0,337	0,337	2,77	2,77	6,73	6,73
Starting amount (pmol)	84,3	84,3	693	693	1681	1681
Inside (pmol)	80,9	72,0	430	453	872	879
Inside (%)	96,0	85,5	62,1	65,4	51,9	52,3
Outside (pmol)	-	-	116	91,8	407	402
Outside (%)	-	-	16,8	13,2	24,2	23,9
Total (pmol)			546	545	1279	1281
% from original			78,9	78,7	76,1	76,2

APPENDIX 5. STELLA® models.

MELANIN BINDING MODELS

Equilibrium binding

One-site binding model



Equations:

Bound(t) = Bound(t - dt) + (Association - Dissociation) * dt
Unbound(t) = Unbound(t - dt) + (Dissociation - Association) * dt

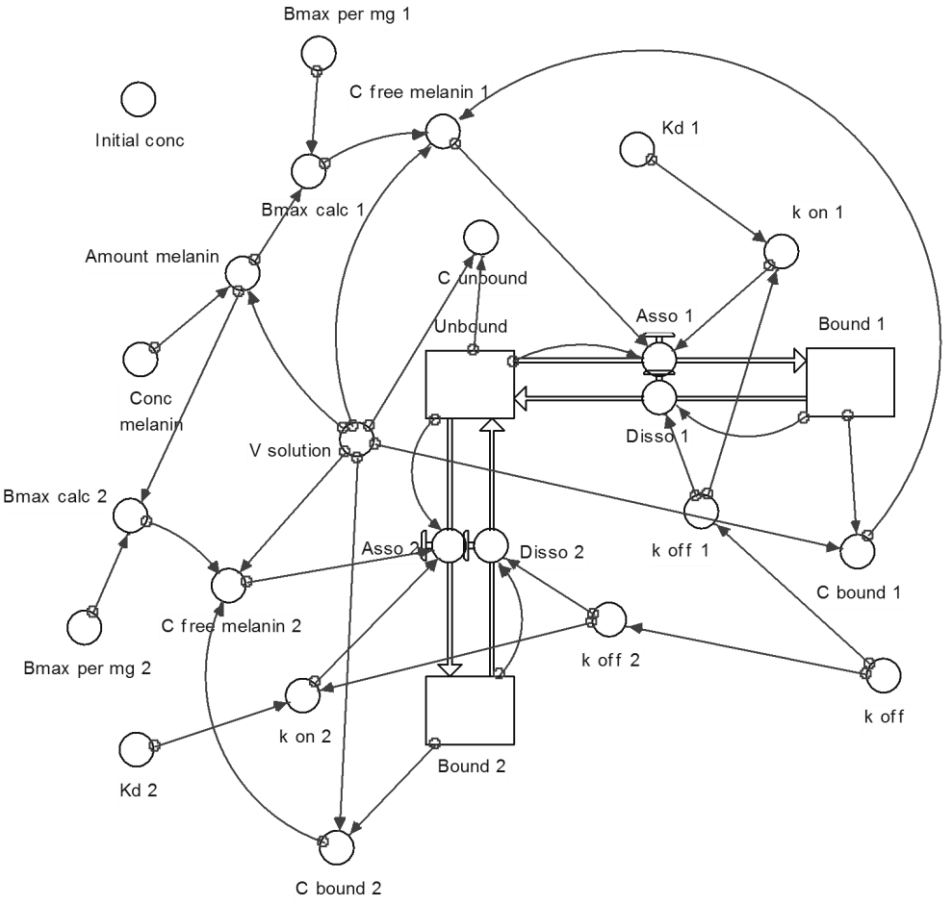
INITIAL Unbound = Initial_conc * V_solution

Association = k_on * C_free_melanin * Unbound
Dissociation = k_off * Bound

Unbound = Initial_conc * V_solution
Amount_melanin = Conc_melanin * V_solution
Bmax_calc = Bmax_per_mg * Amount_melanin
C_bound = Bound / V_solution
C_free_melanin = (Bmax_calc / V_solution) - C_bound
C_unbound = Unbound / V_solution
k_on = k_off / Kd

Conc_melanin = 1000
V_solution = 0.00014

Two-site binding model



Equations:

Bound_1(t) = Bound_1(t - dt) + (Asso_1 - Disso_1) * dt
Bound_2(t) = Bound_2(t - dt) + (Asso_2 - Disso_2) * dt
Unbound(t) = Unbound(t - dt) + (Disso_1 + Disso_2 - Asso_1 - Asso_2) * dt

INITIAL Unbound = Initial_conc * V_solution

Asso_1 = k_on_1 * C_free_melanin_1 * Unbound
Disso_1 = k_off_1 * Bound_1
Asso_2 = k_on_2 * C_free_melanin_2 * Unbound
Disso_2 = k_off_2 * Bound_2

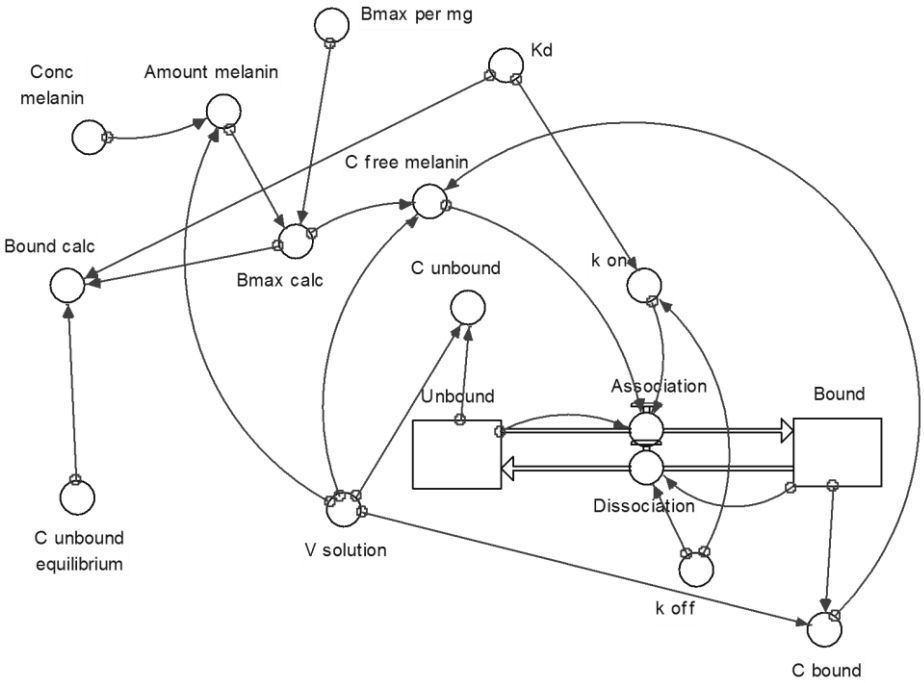
Amount_melanin = Conc_melanin * V_solution
Bmax_calc_1 = Bmax_per_mg_1 * Amount_melanin
Bmax_calc_2 = Bmax_per_mg_2 * Amount_melanin
C_bound_1 = Bound_1 / V_solution
C_bound_2 = Bound_2 / V_solution
C_free_melanin_1 = (Bmax_calc_1 / V_solution) - C_bound_1
C_free_melanin_2 = (Bmax_calc_2 / V_solution) - C_bound_2
C_unbound = Unbound / V_solution

k_off_1 = k_off
k_off_2 = k_off
k_on_1 = k_off_1 / Kd_1
k_on_2 = k_off_2 / Kd_2

Conc_melanin = 1000
V_solution = 0.00014

Dissociation from melanin

One-site binding model

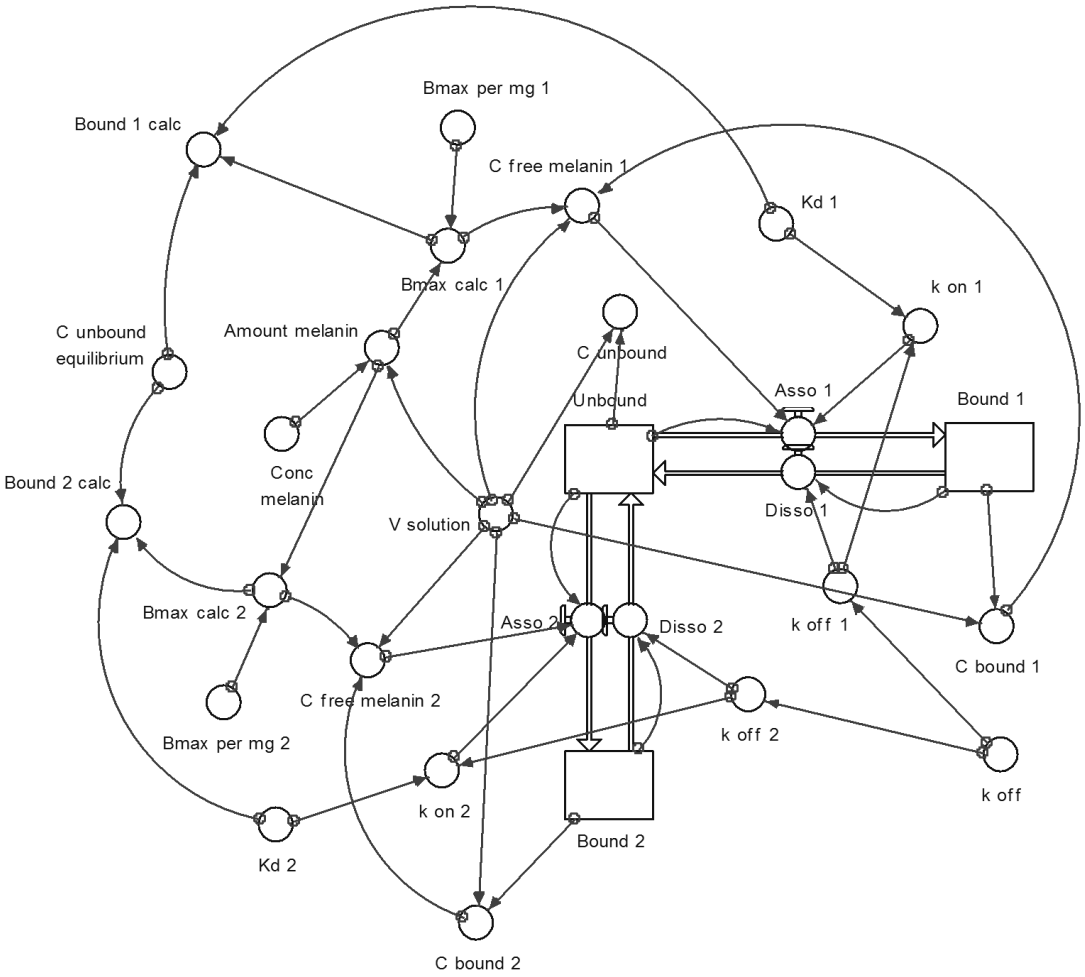


Equations (only the ones that are in addition to the equilibrium binding model):

INITIAL Bound = Bound_calc
INITIAL Unbound = 0

Bound_calc = Bmax_calc * C_unbound_equilibrium / (Kd + C_unbound_equilibrium)

Two-site binding model



Equations (only the ones that are in addition to the equilibrium binding model):

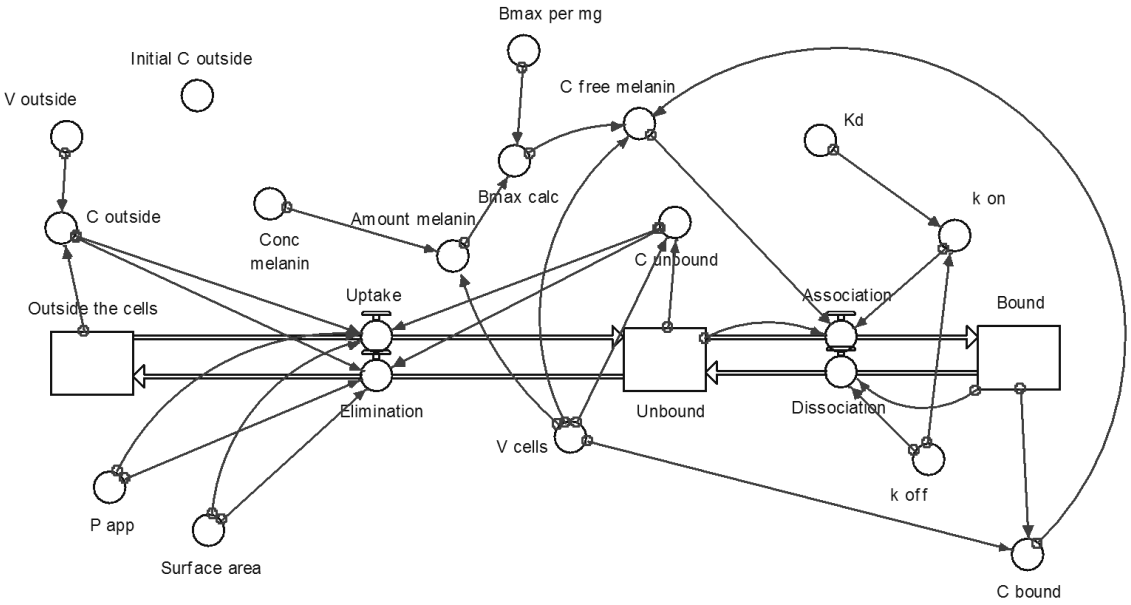
INITIAL Bound_1 = Bound_1_calc
INITIAL Bound_2 = Bound_2_calc
INITIAL Unbound = 0

Bound_1_calc = Bmax_calc_1 * C_unbound_equilibrium / (Kd_1 + C_unbound_equilibrium)
Bound_2_calc = Bmax_calc_2 * C_unbound_equilibrium / (Kd_2 + C_unbound_equilibrium)

CELL MODELS

Uptake

One-site binding model



Equations (only the ones that are in addition to earlier models):

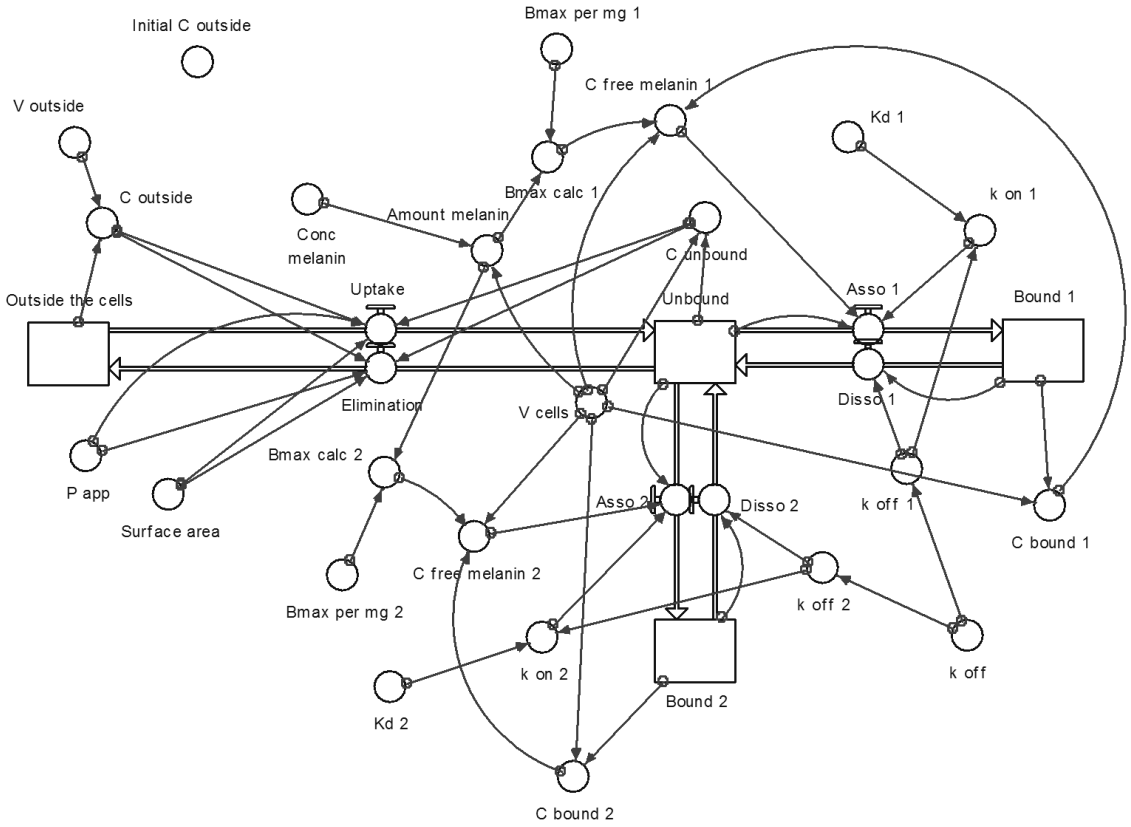
Outside_the_cells(t) = Outside_the_cells(t - dt) + (Elimination - Uptake) * dt
INITIAL Outside_the_cells = Initial_C_outside * V_outside

Elimination = P_app * Surface_area * (C_unbound - C_outside)
Uptake = P_app * Surface_area * (C_outside - C_unbound)
Unbound(t) = Unbound(t - dt) + (Dissociation + Uptake - Association - Elimination) * dt

Amount_melanin = Conc_melanin * V_cells
C_outside = Outside_the_cells / V_outside
C_unbound = Unbound / V_cells

Conc_melanin = 750000
Surface_area = 0.011
V_cells = 0.0000001
V_outside = 0.00025

Two-site binding model



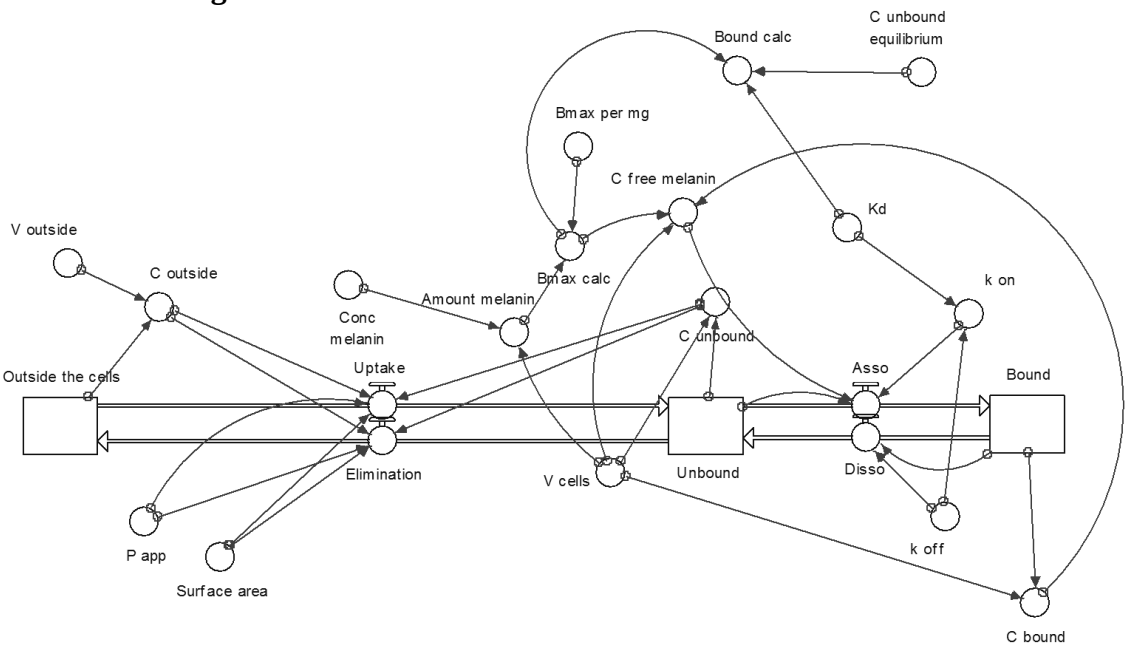
Equations (only the ones that are in addition to earlier models):

Outside_the_cells(t) = Outside_the_cells(t - dt) + (Elimination - Uptake) * dt
INITIAL Outside_the_cells = Initial_C_outside * V_outside

Unbound(t) = Unbound(t - dt) + (Disso_1 + Disso_2 + Uptake - Asso_1 - Asso_2 - Elimination) * dt

Elimination

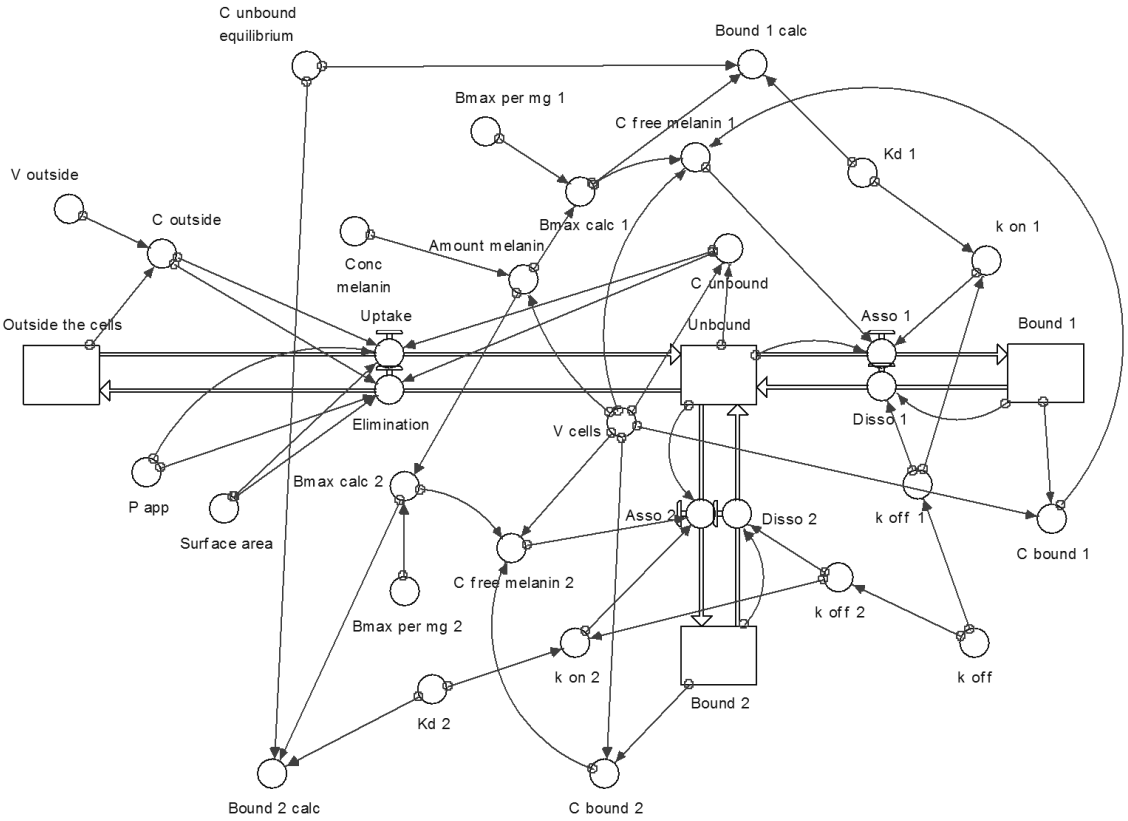
One-site binding model



Equations (only the ones that are in addition to earlier models):

INITIAL Bound = Bound_calc
INITIAL Outside_the_cells = 0
INITIAL Unbound = 0

Two-site binding model



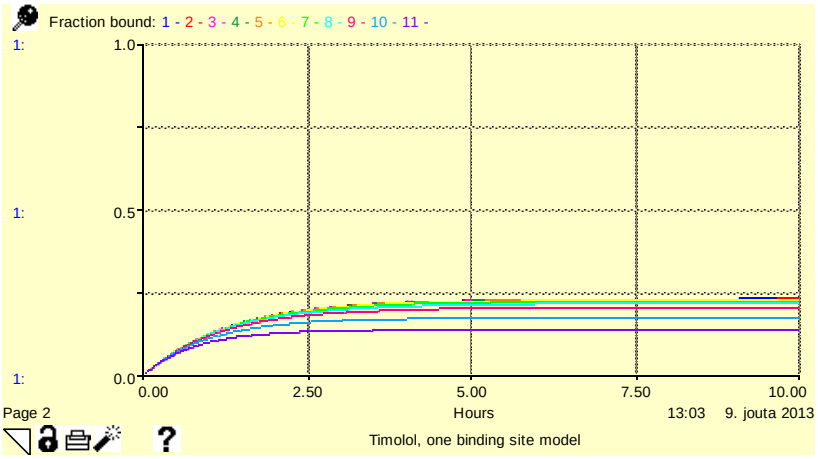
Equations (only the ones that are in addition to earlier models):

INITIAL Bound_1 = Bound_1_calc
INITIAL Bound_2 = Bound_2_calc
INITIAL Outside_the_cells = 0
INITIAL Unbound = 0

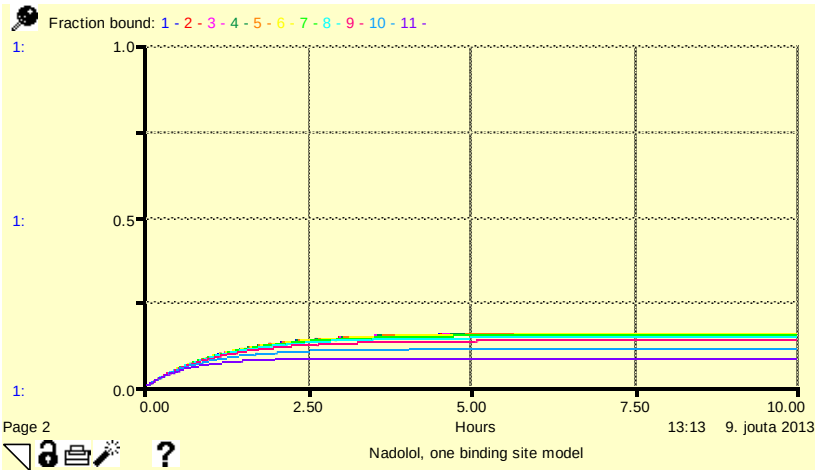
APPENDIX 6. Melanin binding model: time course of binding.

Modeled time course of melanin binding with concentrations 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250 and 500 µM (from 1 to 11 in the graphs, respectively).

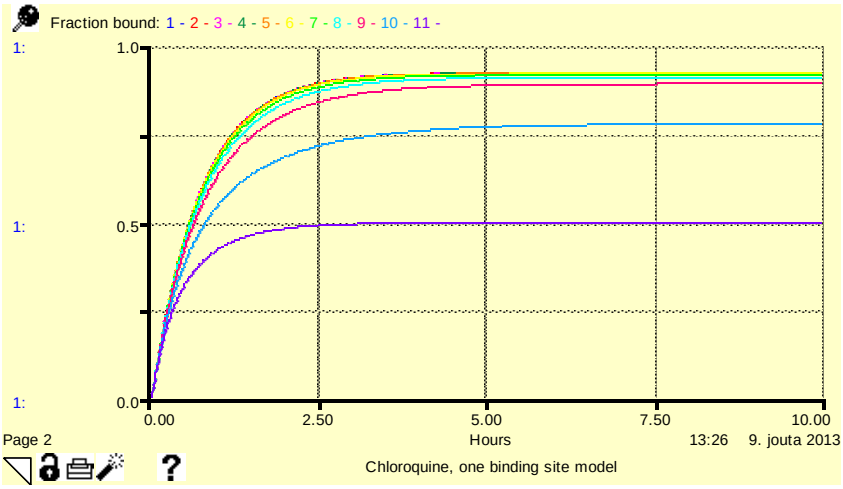
One-site binding model



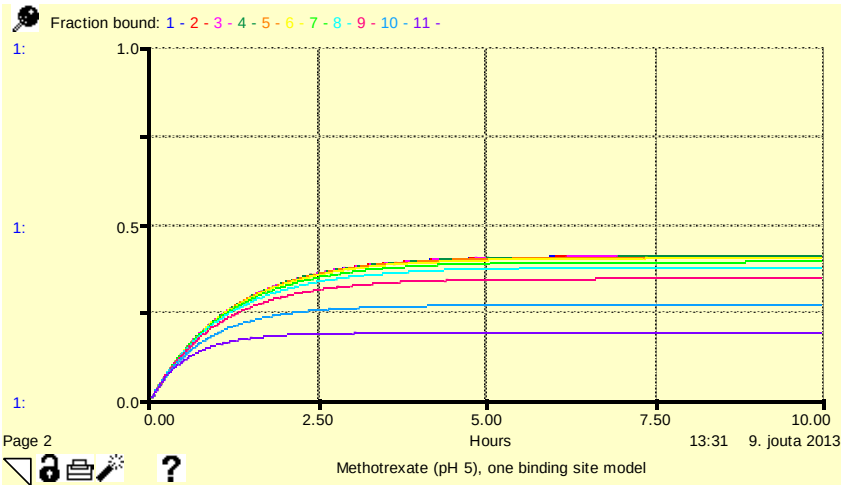
Timolol, k_{off} = 0.6 h^{-1}



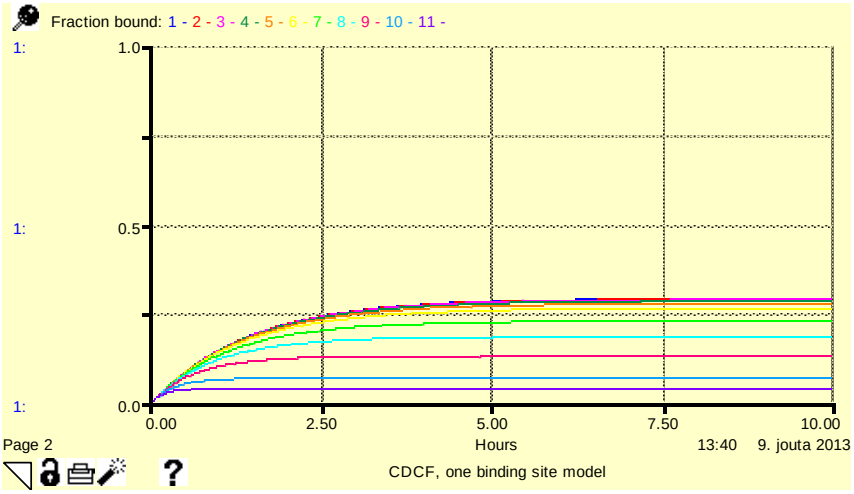
Nadolol, k_{off} = 0.7 h^{-1}



Chloroquine, k_{off} = 0.1 h^{-1}

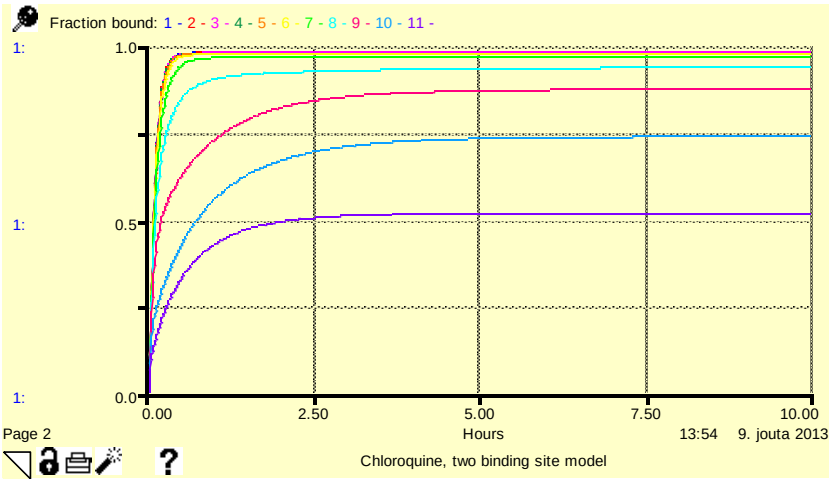


Methotrexate (pH 5), k_{off} = 0.5 h^{-1}

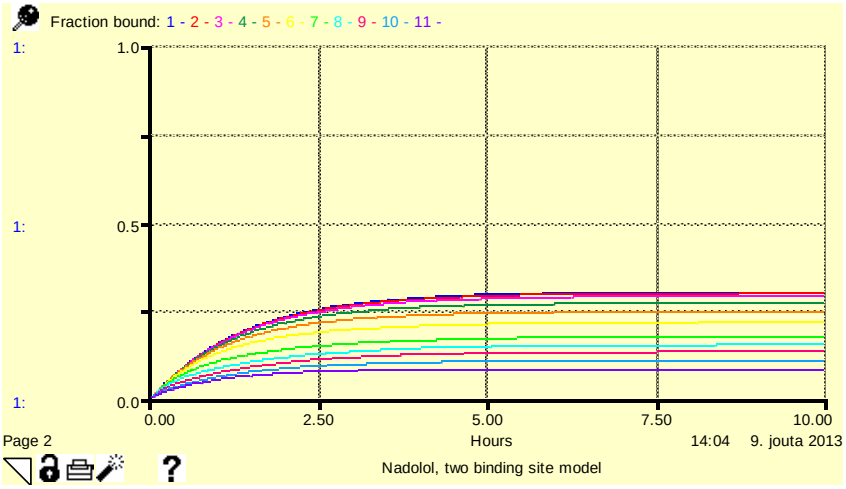


CDCF (pH 5), k_{off} = 0.5 h^{-1}

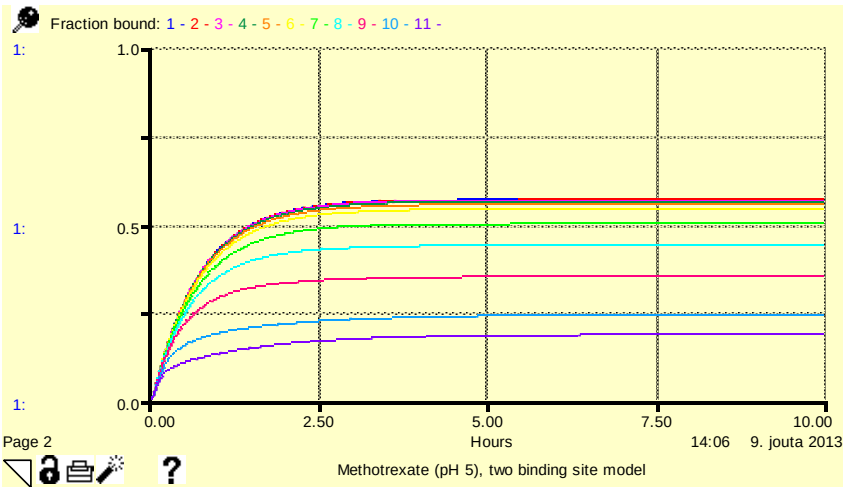
Two-site binding model



Chloroquine, k_{off} = 0.2 h^{-1}



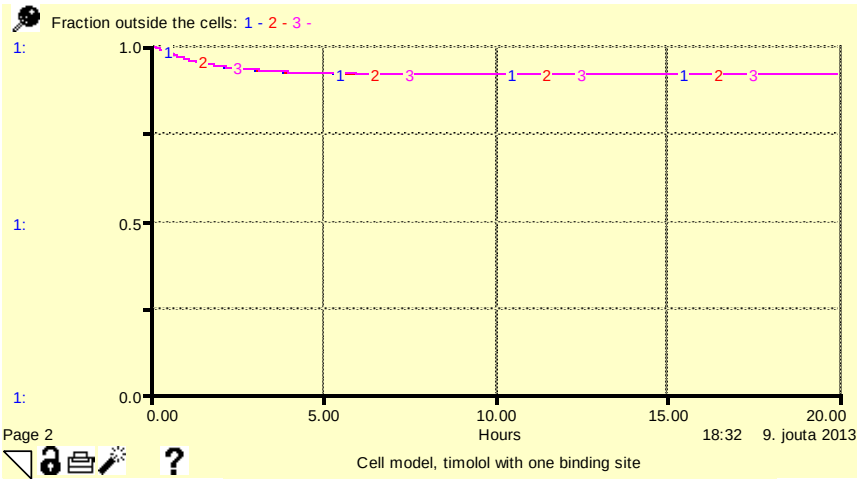
Nadolol, k_{off} = 0.5 h^{-1}



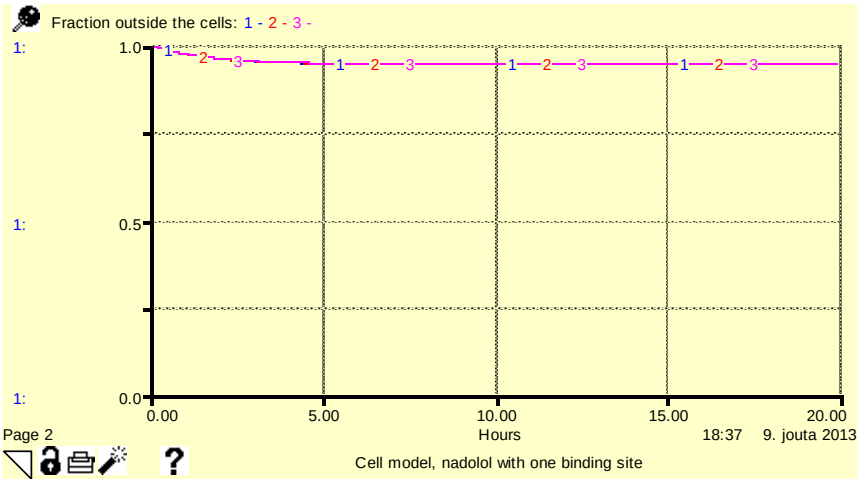
Methotrexate, k_{off} = 0.6 h^{-1}

APPENDIX 7. Cell model: uptake.

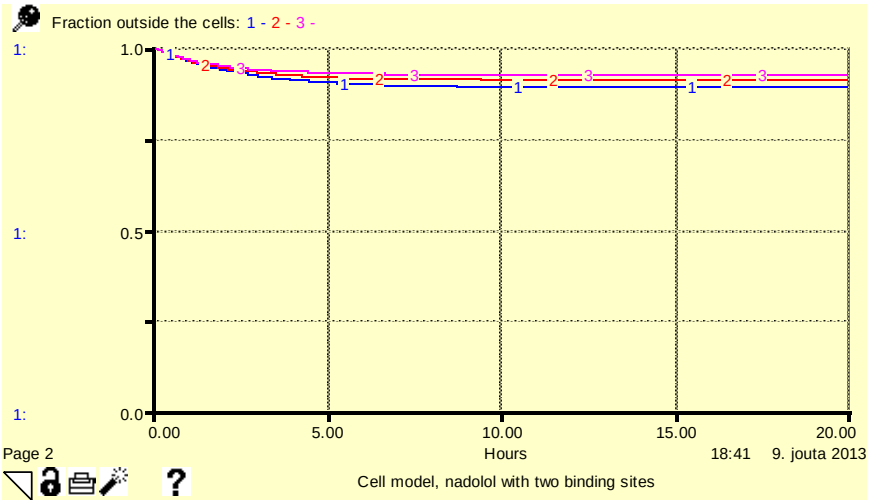
Cell model: fraction outside the cells with starting concentrations 1, 5 and 10 µM (1 to 3 in the graphs, respectively).



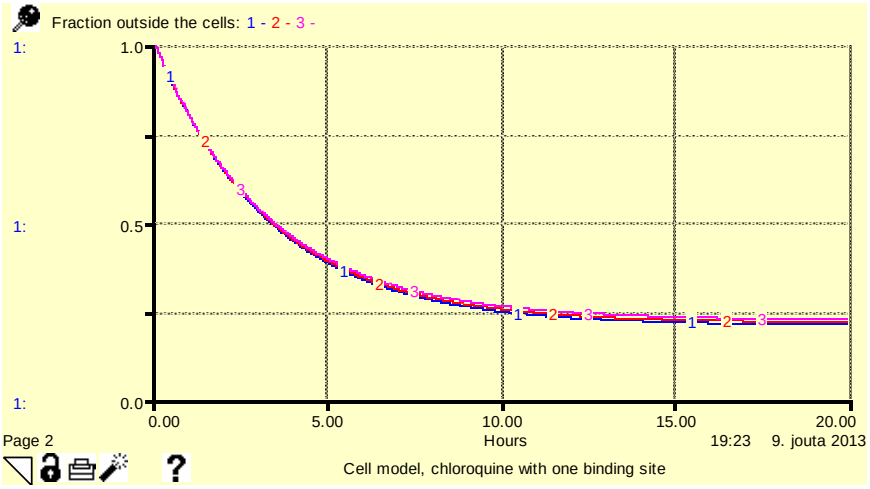
Timolol with one binding site.



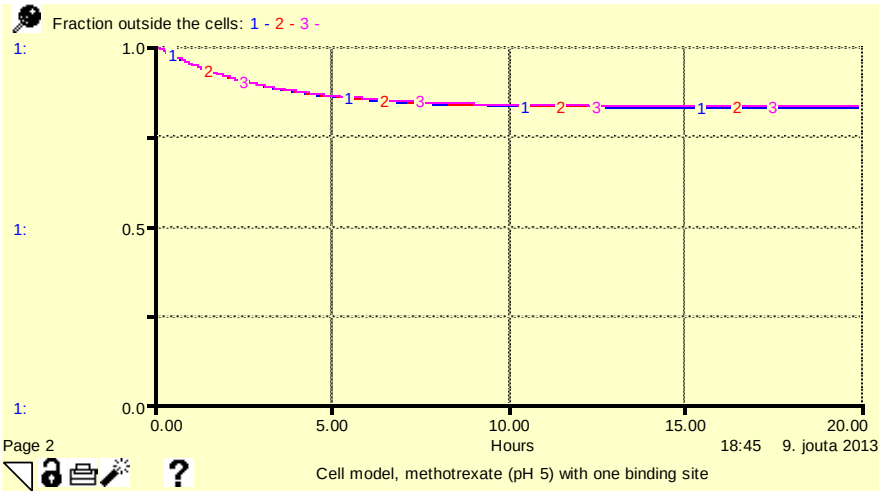
Nadolol with one binding site.



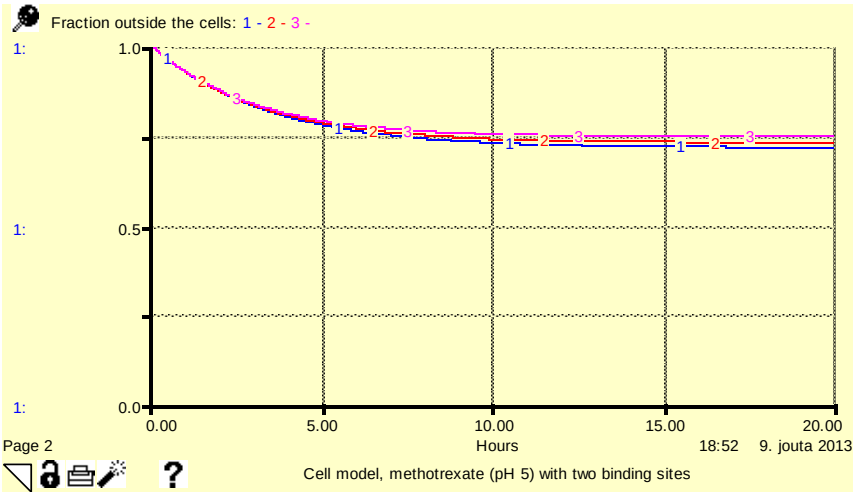
Nadolol with two binding sites.



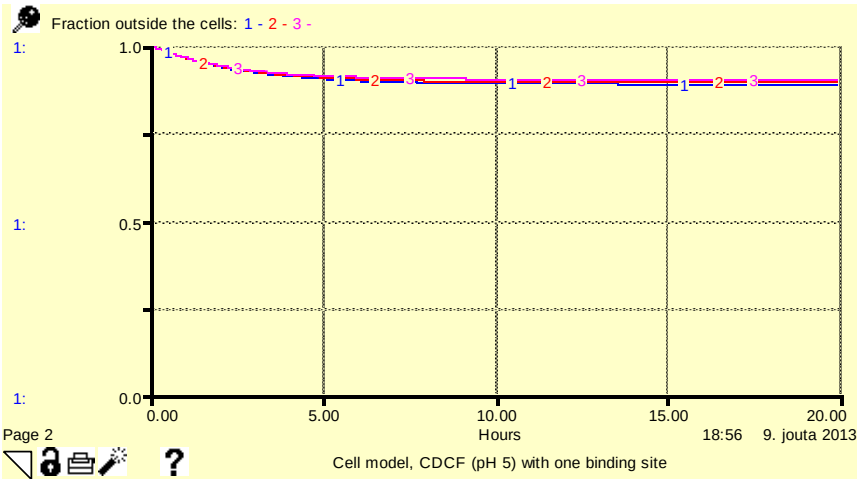
Chloroquine with one binding site.



Methotrexate (pH 5) with one binding site.



Methotrexate (pH 5) with two binding sites.



CDCF (pH 5) with one binding site.