EMMA HEIKKINEN

Ocular drug development aims to overcome frequent dosing and to innovate new drug therapies. Pharmacokinetic and drug delivery aspects pose a challenge for the development. New information on the factors affecting drug pharmacokinetics in the eye would benefit the development of novel ocular drugs and drug delivery systems. This thesis addresses ocular pharmacokinetics and drug delivery in terms of drug metabolism, drug partitioning, drug delivery system design and method development.
PHARMACOKINETIC AND METHODOLOGICAL INSIGHTS INTO OCULAR DRUG DEVELOPMENT
Emma Heikkinen

PHARMACOKINETIC AND METHODOLOGICAL INSIGHTS INTO OCULAR DRUG DEVELOPMENT

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ABSTRACT

Age-related macular degeneration (AMD), diabetic retinopathy, glaucoma and cataract are the leading global causes of vision loss. The wet form of AMD is treated with monthly or bi-monthly intravitreal drug injections, and glaucoma with daily eye drops, both of which burden the patients. Some eye diseases, such as dry AMD and cataract are lacking drug therapies. Thus, there is a clear need for new drugs and drug delivery systems with sustained effects. Pharmacokinetic and delivery aspects pose a challenge to ocular drug development. New information on the factors affecting ocular drug pharmacokinetics would benefit ocular drug development.

In this thesis work, our aim was to gain new insights in ocular pharmacokinetics, and new approaches for the design of drugs and drug delivery systems. Our first aim was to define the esterase activities of porcine and rabbit ocular tissues - an important feature in the design of prodrugs and biodegradable controlled release materials. Our second aim was to develop resource-efficient methods for preclinical pharmacokinetic screening of drug properties. Thirdly, the correlation between the structure and hydrolytic behavior for ocular prodrug candidates was investigated. Our fourth aim was to evaluate drug distribution in the lens, and to simulate the effects of lenticular drug partitioning on topical pharmacokinetics. Finally, we simulated the effects of drug dose, release rate and clearance on the resulting free drug concentrations in the vitreous after application of an intravitreal implant.

We detected significant differences in esterase activities among the ocular tissues and between two species (rabbit, pig) and proposed a method for scaling in vitro enzyme activity to the whole tissue level. Cassette dosing proved useful in studying prodrug hydrolysis in ocular tissues, and supported the concept that the chemical linkers and steric factors around the cleaving bond affect prodrug hydrolysis. Partitioning of various drugs to the lens was low, and based on imaging mass spectrometry, most drugs distributed only to the surface of the lens. The pharmacokinetic simulations indicated that drug partitioning to the lens does not influence drug concentrations in the aqueous humor. Finally, pharmacokinetic simulations revealed that drug clearance from vitreous increases the requirements of drug potency and drug load in intravitreal implants.

The methods and data of this study may be beneficial in the development of novel drugs and delivery systems for ophthalmic use.

Keywords: drug development, drug metabolism, intravitreal injection, ocular pharmacokinetics, prodrug
TIIVISTELMÄ


Työn tuloksiä ja menetelmiä voidaan hyödyntää silmälääkkeiden ja lääkkeensäattomenetelmien kehityksessä.

Avainsanat: aihiolääkkeet, farmakokinetiikka, lääkeainemetabolia, lääkesuunnittelu, silmä, silmäaudit
Luokitus: QV 38, QV 745, QV 785, WB 340

Yleinen suomalainen ontologia: lääkeaineet; lääkkeet; lääkesuunnittelu; aihiolääkkeet; farmakokinetiikka; aineenvaihdunta; hydrolyysi; annostelu; injektiot; silmät; silmätaudit
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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications:


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### ABBREVIATIONS

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<tr>
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<tbody>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>CL_{ivt}</td>
<td>Intravitreal clearance</td>
</tr>
<tr>
<td>DME</td>
<td>Drug metabolizing enzyme</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme classification</td>
</tr>
<tr>
<td>HPLC-MS/MS</td>
<td>High-performance liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>K_{p}</td>
<td>Partition coefficient</td>
</tr>
<tr>
<td>logD_{7.4}</td>
<td>Logarithm of octanol-water distribution coefficient at pH 7.4</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>PSA</td>
<td>Polar surface area</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>t_{1/2}</td>
<td>Half-life</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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1 INTRODUCTION

Vision is certainly one of the most important of a human being’s five senses. It is crucial for living a normal daily life and the loss of sight and blindness severely decreases a person’s ability to perform daily tasks and impacts negatively on psychological well-being. In 2015, globally 217 million people suffered from moderate or severe vision impairment, and 36 million from acquired blindness (Flaxman et al., 2017). The leading causes of vision impairment and blindness in industrialized countries are age-related macular degeneration (AMD), glaucoma and diabetic retinopathy, whereas in low-income countries, cataract is the most common cause of visual loss (Flaxman et al., 2017). In the forthcoming decades, the global prevalence of these diseases is expected to grow as populations age (Flaxman et al., 2017). In addition, there are rare diseases such as retinitis pigmentosa and stargardt disease with a low prevalence in the population, yet also leading to vision loss. Dry eye disease is common ocular disease that does not cause blindness, but may cause significant discomfort, pain and visual dysfunction due to pathological changes in the tear film and ocular surface (Akpek & Smith, 2013b).

Diseases affecting the posterior segment of the eye, such as AMD and diabetic macular edema, are treated with intravitreal injections. AMD is the leading cause of vision loss in the industrialized countries, and the number of patients is growing rapidly in their aging populations (Akpek & Smith, 2013b; Wong et al., 2014). In the two forms of AMD (dry, wet), the part of the retina responsible for the central vision, the macula, is affected. The dry form (geographic atrophy) advances gradually over several years. Dry AMD (80-90% of all AMD patients) do not have any approved pharmaceutical treatment (Al-Zamil & Yassin, 2017). In wet AMD, abnormal growth of choroidal vessels takes place and this form of these diseases progresses more aggressively. The neovascularization in wet AMD is linked to upregulation of vascular endothelial growth factor (VEGF) and is treated with injections of VEGF-inhibitor drugs into the vitreous (Al-Zamil & Yassin, 2017; Singh et al., 2019). Similar to wet AMD, VEGF plays a role in diabetic retinopathy, which is also treated with intravitreal anti-VEGF injections in order to control neovascularization of the retinal capillaries (Akpek & Smith, 2013a; Heng et al., 2013). Nonetheless, chronic treatment with anti-VEGF injections in AMD and diabetic retinopathy is a burden to patients and healthcare systems. Each year, it is estimated that 20 million intravitreal injections are administered.

In global terms, glaucoma is the leading cause for irreversible blindness (E. Chang & Goldberg, 2012). In glaucoma, the retinal ganglion cells degenerate progressively, which leads to vision loss. Currently the only way to slow the vision loss is to reduce the patients’ intraocular pressure with medications, such as beta-adrenergic blockers or prostaglandin analogues (Akpek & Smith, 2013a). The drugs are dosed as eyedrops once a day, however less than 50% of patients adhere to the drug treatment (McClelland et al., 2019). Moreover, some patients show optic disc
changes despite lowering of their intraocular pressure (Chang & Goldberg, 2012; Cursiefen et al., 2019).

Unfortunately, the current treatment options for ocular diseases have serious drawbacks. The chronic nature of the treatments in wet AMD, diabetic retinopathy and glaucoma constitute a burden to the patients. Moreover, some eye diseases, such as dry AMD, glaucomatous optic disc changes and cataract currently lack drug therapies. Therefore, there is an urgent need for the development of new therapies.

The development of new ocular drug therapies is hindered by drug delivery to the target: eye anatomy and physiology are complex and there are various barriers that restrict drug entry into the eye (Gaudana et al., 2010). Because of these barriers, reaching ocular tissues with systemic dosing (e.g. tablets) is difficult without exposing the rest of the body to high drug concentrations, which might cause adverse effects. Therefore, local drug administration, such as dosing with eye drops or intravitreal injection, is usually used to achieve adequate drug exposure in the anterior or the posterior ocular segment, respectively. In particular, retinal drug delivery is a challenge, since it is impossible to achieve sufficient drug exposure in the retina with topical dosing. Therefore, invasive drug administration techniques, such as intravitreal injections, are needed to treat AMD and diabetic retinopathy patients.

Ocular drug delivery and pharmacokinetics contribute substantially to ocular drug research and development for both the anterior and posterior segment diseases. The pharmacokinetics of ocular drugs are rather complex (Maurice & Mishima, 1984), and various factors affect the absorption, distribution, metabolism and excretion of ocular drugs. Nevertheless, these factors are not yet understood in adequate detail. This thesis work aims to generate novel information on the factors that affect pharmacokinetics after topical and intravitreal drug administration. Furthermore, prodrug and formulation approaches were investigated in the ocular drug delivery context.
2 REVIEW OF THE LITERATURE

2.1 OCULAR PHARMACOKINETICS

The eye is protected from topical and systemic drugs by various physiological barriers (Maurice & Mishima, 1984). These barriers are a challenge in ocular drug delivery, since most ocular drug targets are located in the inner parts of the eye. In principle, targets in the anterior eye segment, such as conjunctiva, cornea, iris and ciliary body (Figure 1) can be reached with topical drug dosing, for example with eye drops. This administration route is commonly used for the treatment of corneal and conjunctival inflammation, glaucoma and dry eye disease. In contrast, tissues in the posterior eye segment, such as retina, retinal pigment epithelium (RPE) and choroid (Figure 1) are practically impossible to reach with topical dosing. Therefore, more invasive drug dosing, such as intravitreal injection, is required in the treatment of AMD and diabetic retinopathy. The anatomy of the eye and the most common drug administration routes, topical and intravitreal dosing, are illustrated in Figure 1. Drug distribution and elimination routes and ocular barriers are shown in Figure 2.

![Figure 1. Ocular anatomy and topical and intravitreal drug dosing. Structure of neural retina, RPE and choroid with the limiting membranes are illustrated in more detail in the inset.](image-url)
2.1.1 Topical administration

Topical drug dosing e.g. with eye drops, has been used in the clinics for decades. After eye drop administration, a large fraction of the dose ends up in the nasolacrimal duct, and further to the nose, gastrointestinal tract and systemic circulation (Figure 2) (Järvinen et al., 1995; Patton & Robinson, 1976; Sigurdsson et al., 2007). The remaining drug can be absorbed into the cornea, but the tight junctions between corneal epithelial cells limit drug permeation (Pescina et al., 2015; Ramsay et al., 2018). Furthermore, the conjunctiva poses a permeability barrier for drug absorption (Huang et al., 1989), although to a lesser extent than the cornea due to its leakier epithelia and larger surface area (Ramsay et al., 2017). The major fraction of the drug absorbed to conjunctiva is eliminated through blood flow to the systemic blood circulation (Figure 2) (Sigurdsson et al., 2007). Overall, typically more than 50% of the instilled dose enters the systemic circulation (Urtti & Salminen, 1993).

Corneal and conjunctival barriers and precorneal drug loss limit drug penetration from the tear fluid into the inner eye tissues (Figure 2). This can be overcome to some extent with optimal drug properties, such as small size, adequate lipophilicity and low hydrogen bonding capacity (Ramsay et al., 2017; Ramsay et al., 2018), but even in those cases, ocular bioavailability can be as low as 5% (Maurice & Mishima, 1984; Urtti et al., 1990). For example, corneal and aqueous humor concentrations of timolol were 60- and 500-fold lower, respectively, than in the tear fluid after an instillation of a single eyedrop to rabbits (Urtti et al., 1990). For macromolecular drugs, such as antibodies, the permeability across the cornea is very low – for 30-50 kDa molecules the ex vivo penetration was in the 0.01-1% range.
(Brereton et al., 2005). The conjunctiva also restricts the permeation of macromolecules, though compounds up to 20-40 kDa have been reported to cross conjunctiva ex vivo (Huang et al., 1989).

After reaching the anterior chamber, the drug may be eliminated through aqueous humor outflow or be distributed from aqueous humor into ciliary body, iris and the lens (Figure 2). In the ciliary body and iris, drugs can distribute to the local capillaries and be eliminated via the systemic circulation. The iris-ciliary body includes the blood-aqueous barrier that limits drug passage from the systemic circulation to the eye and vice versa (Raviola, 1977). The lens hinders drug diffusion from the aqueous humor to the vitreous (Christoforidis et al., 2013; Green et al., 1983); drug distribution to the lens is reviewed in more detail in 2.1.3.

Topically dosed drugs can reach target tissues mainly in the anterior segment of the eye, but the concentrations in the posterior eye segment (e.g. retina, choroid) are negligible (Acheampong et al., 1995; Araie et al., 1982; Sigurdsson et al., 2005; Sigurdsson et al., 2007; Urtti et al., 1984; Wang et al., 2019). For example, the drug concentrations in the posterior tissues of rabbits are typically 5-20 fold lower than in the aqueous humor (Sigurdsson et al., 2005; Sigurdsson et al., 2007; Urtti et al., 1990). In preclinical animal species, with topical dosing, a significant part of the drug in the posterior tissues originates from the systemic circulation (Sigurdsson et al., 2007). Therefore, topical administration is only suitable for the treatment of diseases affecting the anterior segment of the eye and intravitreal administration is needed for the treatment of posterior eye diseases.

2.1.2 Intravitreal administration

After intravitreal administration, the drug distributes from the vitreous in the administration site, towards the surrounding tissues (Figure 1, Figure 2). Vitreous is an avascular, clear gel-like tissue comprising mostly of water, collagen and hyaluronic acid (Bishop, 2000; Schepens & Neetens, 1987). Usually, vitreous does not hinder the diffusion of small molecular weight drugs or macromolecules, but it represents a significant barrier to the diffusion of particles over 500 nm in size (Käsdorf et al., 2015; Tan et al., 2011; Xu et al., 2013) and positively charged smaller particles (Käsdorf et al., 2015). Some small molecular weight drugs can bind to some extent to the vitreous in vitro (maximally ≈70%, usually < 30%), but this binding seems to exert only a modest extent on the vitreal pharmacokinetics (Rimpelä et al., 2018).

Inner and outer limiting membranes isolate the retina from vitreous and photoreceptors (Figure 1). Their barrier roles for small molecule drugs are insignificant, but they hinder the permeation of macromolecules and nanoparticles to the retina (Bourges et al., 2003; Jackson et al., 2003; Julien et al., 2014; Kamei et al., 1999; Marmor et al., 1985; Pitkänen et al., 2004; Yu et al., 2001). In the retina, the retinal capillary endothelia hinders the permeation of macromolecules and particles from the retina to systemic blood and vice versa (Smith & Rudt, 1975; Törnquist et al., 2018).
1990), but it does allow the passage of lipophilic small molecular weight compounds (Tachikawa et al., 2010; Thornit et al., 2010).

RPE is a monolayer of cells located between the photoreceptor layer and choroid (Figure 1); there are tight junctions between the cells (Figure 2) (Rizzolo et al., 2011), limiting the permeation of drugs from vitreous to systemic circulation and vice versa (Pitkänen et al., 2005; Ramsay et al., 2019). Hydrophilic small molecular weight drugs have about 5- to 10-fold lower permeability across RPE than lipophilic drugs, and macromolecules 100- to 500-fold lower permeability than small molecular weight drugs (Mannermaa et al., 2010; Pitkänen et al., 2005; Ramsay et al., 2019). Active drug transporters have been detected in RPE cell models (Hellinen et al., 2019; Mannermaa et al., 2006; Pelkonen et al., 2017b) and they could affect drug distribution between vitreous and systemic circulation. The functional impact of these transporters on ocular drug pharmacokinetics is not well understood, although for some hydrophilic compounds, the role of active transport seems to be substantial (Cunha-Vaz & Maurice, 1967). Together with the retinal capillaries, the RPE forms the blood-retinal barrier (Raviola, 1977), which prevents the entry of xenobiotics from the systemic circulation into the eye (Toda et al., 2011). Posterior to the RPE, the vascular choroid connects the posterior eye segment with the systemic blood circulation (Figure 1, Figure 2). The role of choroid in drug elimination is related to the high blood flow (43 ml/h in humans (Schmetterer & Kiel, 2012)), which clears the drugs to the systemic circulation.

Some ocular tissues, such as RPE, choroid and iris-ciliary body contain melanin pigment. Many drugs bind to melanin (Araie et al., 1982; Aula et al., 1988; Báthory et al., 1987; Boman, 1975; Farah & Patil, 1979; Hayasaka et al., 1988; Pelkonen et al., 2017a; Salazar & Patil, 1976; Salminen & Urtti, 1984; Shimada et al., 1976), which can increase the drug’s retention time and prolong the drug’s therapeutic effect in the pigmented tissues (Araie et al., 1982; Boman, 1975; Salazar & Patil, 1976; Salminen & Urtti, 1984).

The elimination from the vitreous can be divided into anterior and posterior elimination (Maurice & Mishima, 1984). Anterior elimination is governed by drug diffusion from vitreous into aqueous humor and further into the systemic circulation. Anterior elimination contributes to the elimination of both small and large molecular weight drugs (del Amo et al., 2015; Lamminsalo et al., 2018). Posterior elimination consists of drug elimination through the iris-ciliary body and RPE (Maurice & Mishima, 1984). In the iris-ciliary body, the blood-aqueous barrier prevents permeation of molecules from vitreous into the systemic circulation. RPE contributes to posterior elimination to a large extent by regulating the passage of drugs to choroidal blood flow (Pitkänen et al., 2005; Ramsay et al., 2019). Small molecular weight drugs are eliminated from the vitreous mostly via the posterior route.

The two elimination routes from the vitreous complement each other and their relative contributions, as well as the total intravitreal clearance (CLvit) and consequent impact on drug retention time in the vitreous, depend largely on the drug’s properties, mainly size. Macromolecules, which are eliminated mainly
through the anterior route, have a low clearance (CL_{ivt} \approx 0.01-0.1 \text{ ml/h in vivo in rabbit}) and long half-lives (t_{1/2} \approx 1-6 \text{ days in vivo in rabbit}) (Caruso et al., 2020; del Amo et al., 2015; García-Quintanilla et al., 2019). Polymeric structures such as nanoparticles and their degradation products and protein drugs exceeding 2 nm size cannot permeate through the blood-ocular barriers (Ashton & Cunha-Vaz, 1965; Smith & Rudt, 1975; Törnquist et al., 1990), thus they presumably favor the anterior route. Nanoparticles show size- and charge-dependent retention in vivo in the rabbit vitreous with elimination times ranging from days to months (Raju et al., 2012; Sakurai et al., 2001). Intravitreally administered small molecular weight drugs are eliminated more quickly in vitreous (CL_{ivt} = 0.05-1.5 \text{ ml/h, } t_{1/2} = 1-24 \text{ h in vivo in rabbit}) (del Amo et al., 2015). Hydrophilic small molecular weight drugs have lower clearances than lipophilic drugs (50-fold range in CL_{ivt}) (del Amo et al., 2015) because of their impaired permeation across blood-ocular barriers (Pitkänen et al., 2005). High clearance, short half-life and short retention time in the vitreous pose a challenge for drug delivery to the retina.

### 2.1.3 Drug partitioning to the lens

The lens is located between the vitreous and aqueous humor (Figure 1, Figure 3). The lens is suspended from the ciliary muscles with zonules that attach to the lens capsule, which envelopes the lens epithelium and fibers (Dai & Boulton, 2018) (Figure 3). The lens epithelium is located in the anterior lens as a monolayer (Figure 3) and it possesses tight and adherens junctions (Unakar et al., 1991). During lens aging, the epithelial cells migrate towards the lens equator and elongate into lens fibers, which over time form an onion-like structure (Augusteyn, 2010) (Figure 3). The young lens fibers have a softer consistency, and form the lens cortex, whereas the dense, tightly packed old lens fibers make up the lens nucleus (Dai & Boulton, 2018). The water, protein and lipid composition of the lens is unique: 65% of total lens wet weight is water, 34% protein and 1% other compounds (e.g. lipids) (Dai & Boulton, 2018). The composition varies slightly between lens cortex and nucleus; the cortex has a higher water and a lower protein and lipid content than the nucleus. In contrast, the water and protein contents in non-ocular tissues are 75-85% and about 20%, respectively (Forbes et al., 1953; Pethig & Kell, 1987).
The lens hinders the diffusion of drugs between vitreous and aqueous humor in topical and intravitreal dosing (Figure 2), as confirmed in lensectomied rabbits (Christoforidis et al., 2013; Green et al., 1983). The lens is thought to be mainly a physical barrier for drug distribution. Drug binding to the lens can however have relevance in ocular pharmacokinetics and pharmacology. In principle, binding could alter the drug exposure in aqueous humor or vitreous: binding can decrease the free drug concentration or result in the formation of a drug depot. The binding, as well as the spatial drug distribution inside the lens, is also relevant for drugs that have pharmacological activity (e.g. antioxidants, chaperones, N-acetylcarnosine) (Abdelkader et al., 2015; Makley et al., 2015; Thrimawithana et al., 2018) or toxicity (e.g. corticosteroids (Bilgihan et al., 1997; Li et al., 2008) in the lens.

There are a few reports of drug binding to the lens (Table 1). For some drugs, very low concentrations are found in the lens after topical dosing in vivo: for timolol and dexamethasone, 15-fold lower concentrations have been detected in the lens than in the aqueous humor in vivo in rabbits with a single topical dose (Schmitt et al., 1980; Sigurdsson et al., 2007; Urtti et al., 1990). However, some compounds, such as an aldose reductase inhibitors AD-5467 and CT-112, show high partitioning to the lens in vitro (lens-buffer partition coefficient $K_p > 8$) (Ohtori et al., 1991). Most of the studies have been conducted on rabbit lenses with incubation times ranging from 2 to 24 h (Table 1). The short incubations (2-4 h) have mostly yielded $K_p$ values below 1, while in long incubations, 24 h incubations $K_p$ values have ranged from 0.6 to 10. In general, the reports vary in incubation times and tissue handling, and often lack detailed descriptions of important methodological issues such as tissue integrity during...
incubation. Moreover, the effect of drug binding to the lens on aqueous humor or lens drug exposure has not been yet explored in the literature.

Table 1. In vitro lens-buffer partition coefficients (\(K_p\)) and experimentally derived logarithmic octanol-water partition coefficients (\(\log P\)) for various drugs. \(\log P\) not shown for the amino acids or peptides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Species</th>
<th>(K_p)</th>
<th>(\log P)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD-S5467 (aldose-reductase inhibitor)</td>
<td>Rat</td>
<td>4-10* (various concentrations, 24 h incubation)</td>
<td>1.00</td>
<td>Ohtori et al., 1991</td>
</tr>
<tr>
<td>Anthracene</td>
<td>Rabbit</td>
<td>4 (24 h incubation)</td>
<td>4.5</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Arginine vasopressin</td>
<td>Rabbit</td>
<td>1.6 for capsule, 0.7 for lens body (24 h incubation)</td>
<td>-</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Bupironol</td>
<td>Rabbit</td>
<td>1 (24 h incubation)</td>
<td>2.4</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Human, rabbit</td>
<td>In human 0.3 and in rabbit 0.5* (2 h incubation)</td>
<td>1.14**</td>
<td>Heyrman et al., 1989</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>Rabbit</td>
<td>0.6 (24 h incubation)</td>
<td>0.4</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Clonidine</td>
<td>Rabbit</td>
<td>0.7 (24 h incubation)</td>
<td>1.4</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>CT-112 (aldose-reductase inhibitor)</td>
<td>Rat</td>
<td>Approximately 8-15* (24 h incubation, various concentrations)</td>
<td>2.65</td>
<td>Ohtori et al., 1991</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Rabbit</td>
<td>For capsule and cortex approximately 5 (24 h incubation)</td>
<td>-</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Human, rabbit</td>
<td>In human 0.3 and in rabbit 0.6* (2 h incubation)</td>
<td>1.83**</td>
<td>Heyrman et al., 1989</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>Rabbit</td>
<td>10 (24 h incubation)</td>
<td>5.1</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>Human, rabbit</td>
<td>In human and rabbit 0.2* (2 h incubation)</td>
<td>-1.37***</td>
<td>Heyrman et al., 1989</td>
</tr>
<tr>
<td>Fluorometholene</td>
<td>Rabbit</td>
<td>1 (24 h incubation)</td>
<td>2.1</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Rabbit</td>
<td>For capsule approximately 10 and for lens body approximately 15 (24 h incubation)</td>
<td>-</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Hexamethylene lauramide</td>
<td>Rabbit</td>
<td>7 (24 h incubation)</td>
<td>7.3</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Padimate-O</td>
<td>Rabbit</td>
<td>10 for capsule, 1 for cortex and 0.4 for nucleus (24 h incubation)</td>
<td>6.6</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Parsol-1789</td>
<td>Rabbit</td>
<td>2 (24 h incubation)</td>
<td>6.7</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Human, rabbit</td>
<td>In human 0.3 and in rabbit 0.6* (2 h incubation)</td>
<td>1.1***</td>
<td>Heyrman et al., 1989</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Rabbit</td>
<td>7 for capsule, 6 for cortex and 0.5 for nucleus (24 h incubation)</td>
<td>3.9</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Proline</td>
<td>Rabbit</td>
<td>&lt;2 (24 h incubation)</td>
<td>-</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Serine</td>
<td>Rabbit</td>
<td>For capsule &gt;0, for cortex 20 and for nucleus 10 (24 h incubation)</td>
<td>-</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Sulfacetamide</td>
<td>Rabbit</td>
<td>0.9 for capsule, 0.3 for lens body (24 h incubation)</td>
<td>-1.0</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Rabbit</td>
<td>7 (24 h incubation)</td>
<td>3.3</td>
<td>Tang-Liu et al., 1992</td>
</tr>
<tr>
<td>Timolol</td>
<td>Rabbit</td>
<td>0.7* (4 h incubation)</td>
<td>1.83</td>
<td>Ahmed et al., 1989</td>
</tr>
</tbody>
</table>

* \(K_p\) value calculated from the experimental data  
** experimental value from Pyka et al., 2006  
*** experimental value from Hansch et al., 1998
2.1.4 Drug metabolizing enzymes in the eye

Drug metabolizing enzymes (DMEs) contribute to drug pharmacokinetics and toxicity. Intuitively, it would be predicted that in ocular tissues the expression of DMEs would be in general much lower than in the liver. However, ocular DMEs are important since they can metabolize and inactivate ophthalmic drugs, which may lead to formation of eye-specific metabolites and organ-specific toxicity. Many clinically relevant drugs, such as chloramphenicol (Shimada et al., 1988), betaxolol (Bushee et al., 2015), levobunolol (Argikar et al., 2016; Lee et al., 1988; Tang-Liu et al., 1988), morphine (Watkins et al., 1991), sulindac (Shimada et al., 1988) and tafluprost (Fukano & Kawazu, 2009) undergo metabolism in the anterior ocular tissues. Ocular DMEs also have an important role in the bioconversion of prodrugs (Barot et al., 2012).

In principle, drug metabolism can be divided into two phases. Phase I enzymes add reactive and polar groups with oxidation, reduction and hydrolysis reactions, and make the substrate more hydrophilic. Phase II enzymes catalyze conjugation of glutathione, sulfate and glucuronic acid, making the substrate usually less pharmacologically active. Several phase I and II DMEs, such as cytochrome P450 (CYP) enzymes (Schwartzman et al., 1987; Zhao & Shichi, 1995), esterases (Ellis, 1971; Lee et al., 1982; Mains et al., 2012) and numerous conjugative enzymes (Watkins et al., 1991) are expressed in ocular tissues. The key literature and findings on ocular phase I and II DMEs are summarized in Table 2 and Table 3, respectively.

In mammals, CYP enzymes are very important for xenobiotic metabolism in the liver, since this superfamily is responsible for most of phase I drug metabolism. However, in the eye, the role of CYP enzymes on drug metabolism is not clear. The early metabolism studies in ocular tissue homogenates with probe substrates concluded that cornea, iris-ciliary body, RPE and retina isolated from porcine, bovine and rabbit eyes did possess some CYP superfamily activity (Kishida et al., 1986; Schwartzman et al., 1987; Shichi & Nebert, 1982) (Table 2). In contrast, based on more detailed messenger RNA (mRNA) studies, the key drug-metabolizing CYP enzymes (1A1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5) are either absent or expressed at very low levels in human, rabbit and monkey cornea, iris-ciliary body and retina-choroid (Kölln & Reichl, 2012; Zhang et al., 2008). CYP1B1 protein has been detected from human iris-ciliary body, vitreous, RPE and choroid (Mirzaei et al., 2017; Zhang et al., 2016). These findings suggest that CYP enzymes do not greatly contribute to drug metabolism in the ocular tissues. Other phase I enzymes including mono- and diamine oxidases, various hydrolases and alcohol dehydrogenases have been detected from ocular tissues of various animal species (Table 2).

Esterases (enzyme classification (EC) 3.1) are phase I hydrolase enzymes that cleave esters into acid and alcohol products in the presence of water (Khojasteh et al., 2011). The most important esterases for drug metabolism in the liver are acetyl- (EC 3.1.1.7) and butyryl-cholinesterases (EC 3.1.1.8, also known as pseudocholinesterases), carboxylesterases (EC 3.1.1.1), and paraoxonases (EC 3.1.1.2, also known as arylesterases) (Khojasteh et al., 2011). These esterase subclasses have
also been found in ocular tissues (Table 2). Many studies have determined esterase activities in ocular tissues with unspecific esterase substrates (Chang & Lee, 1982; Lee et al., 1982; Redell et al., 1983). These investigators found high esterase activities from rabbit corneal and conjunctival epithelia. With the exception of the lens and sclera, acetylcholinesterase activity has been detected in all ocular tissues in rabbit, cat, dog, pigeon, rooster, ox, horse, pig and rat (DeRoetth, 1950; Koelle & Friedenwald, 1950; Lee et al., 1983; Lee, 1983; Lee et al., 1985; Petersen et al., 1965; Sánchez-Chávez et al., 1995). Butyrylcholinesterase activity is present in cornea, iris-ciliary body, aqueous humor, neural retina and RPE in the rabbit and rat (Lee, 1983; Lee et al., 1983; Lee et al., 1985; Sánchez-Chávez et al., 1995). In human eyes, both acetyl- and butyrylcholinesterase have been detected in aqueous humor and vitreous (Appleyard et al., 1991), acetylcholinesterase has been observed in iris-ciliary body, RPE and choroid (Zhang et al., 2016) and carboxylesterase in vitreous and retina (Mirzaei et al., 2017; Skeie et al., 2015; Zhang et al., 2015). The presence of paraoxonases 1 and 2 has been confirmed in the rat lens, corneal epithelium and retina (Marsillach et al., 2008).

The presence of the most important phase II enzymes, such as glutathione S-transferases, UDP-glucuronosyltransferases and N-acetyltransferases, in ocular tissues has been confirmed (Table 3) (Ahmad et al., 1988; Miller et al., 1980; Saneto et al., 1982a; Saneto et al., 1982b; Shichi & Nebert, 1982; Watkins et al., 1991). Glutathione S-transferase activity has been detected in rabbit cornea, iris, retina and choroid (Watkins et al., 1991), in bovine cornea, lens, retina and RPE (Ahmad et al., 1988) and in human aqueous humor, vitreous and retina (Chowdhury et al., 2010; Rosenfeld et al., 2015; Zhang et al., 2015; Zhang et al., 2016). In contrast, mRNA levels of some glutathione-S-transferases and UDP-glucuronosyltransferases were low or undetectable in human cornea (Kölln & Reichl, 2012). UDP-glucuronosyltransferase activity has been detected also in rabbit retina and iris (Watkins et al., 1991) and human vitreous (Loukovaara et al., 2015). N-acetyltransferase activity was evident in vitro in rabbit cornea, iris, choroid and retina (Watkins et al., 1991) and in vivo in rat retina and iris (Miller et al., 1980), and the proteins were detected in human aqueous humor, vitreous and retina (Mirzaei et al., 2017; Rosenfeld et al., 2015; Skeie et al., 2015; Sudha et al., 2017; Zhang et al., 2015). In human cornea, mRNA expression of sulfotransferase 1A1 and N-acetyltransferases 1 and 2 were low or undetectable (Kölln & Reichl, 2012).

So far, most of the exploration of ocular DMEs has focused on the identification, activity and activity modulation of the enzymes in non-clinical animal species. Data on enzymes have been mostly obtained from mRNA expression studies, proteomics and activity assays with probe substrates. These methods however have drawbacks. For example, mRNA levels do not necessarily correlate with the actual protein amounts (Gry et al., 2009), and quantitative proteomic studies on the ocular DMEs are sparse. Moreover, protein quantities do not provide reliable information on whether or not the enzyme is functional. In vitro specific activities in tissue homogenates do not allow an assessment of the metabolic activity at the whole
tissue level. The existing studies have been conducted with various methods in a limited set of tissues, which makes data comparison difficult.

In non-ocular applications, *in vitro* drug metabolism is commonly scaled to the whole tissue level with protein mass per gram of tissue: for example, in drug metabolism studies with human liver microsomes, an average mass of microsomal protein per gram of liver along with total liver mass is used to scale the microsomal intrinsic clearance from an *in vitro* system to a whole liver (Iwatsubo *et al.*, 1997; Zhang *et al.*, 2015). Corresponding scalars have been reported also for other tissues, such as lung, kidney, small intestine and colon (De Kanter *et al.*, 2004; Scotcher *et al.*, 2017). In principle, a similar approach could be applicable to ocular tissues as well, yet it has not been explored in the literature.
Table 2. Key literature on phase I ocular enzymes.

<table>
<thead>
<tr>
<th>Enzyme family</th>
<th>Enzyme</th>
<th>Method</th>
<th>Species</th>
<th>Tissues</th>
<th>Summary of the results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP</td>
<td>CYP-family</td>
<td>Substrate metabolism study <em>in vitro</em></td>
<td>Cow</td>
<td>Cornea, iris, ciliary body, lens, retina, RPE-choroid</td>
<td>Highest activity in ciliary body, some activity in RPE-choroid, low activity in cornea, iris and retina. No activity in lens.</td>
<td>Kishida et al., 1986; Schwartzman et al., 1987; Shichi &amp; Nebert, 1982</td>
</tr>
<tr>
<td></td>
<td>1A2, 3A4, 2C8, 2C9, 2C19, 2D6, 2A6, 2E1, 3A5, 2B6, 1B1 mRNA expression in tissues, proteomics for 1B1</td>
<td>Human</td>
<td>Cornea, iris-ciliary body, retina-choroid, 1B1 vitreous and retina</td>
<td>Very low/absent mRNA levels for all the CYPs in cornea, iris-ciliary body and retina-choroid, except low CYP2C8 and 2A6 levels in retina-choroid. 1B1 protein detected in vitreous, but not in retina.</td>
<td>Mirzaei et al., 2017; Zhang et al., 2008</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diamine oxidase</td>
<td>Substrate metabolism <em>in vitro</em></td>
<td>Cow</td>
<td>Cornea, aqueous humor, vitreous, sclera</td>
<td></td>
<td>Crabbe, 1985</td>
</tr>
<tr>
<td></td>
<td>Beta-glucuronidase</td>
<td>Histochemistry</td>
<td>Human, pig, rat</td>
<td>Cornea, conjunctiva, iris</td>
<td>Positive staining in cornea, conjunctiva and iris in all species.</td>
<td>Coupland et al., 1994</td>
</tr>
<tr>
<td></td>
<td>Aminopeptidases M and A</td>
<td>Histochemistry</td>
<td>Human</td>
<td>Trabecular meshwork (human), cornea, conjunctiva, iris (human, pig, rat)</td>
<td>Moderate staining in trabecular meshwork.</td>
<td>Coupland et al., 1993; Coupland et al., 1994</td>
</tr>
<tr>
<td>Peptidases</td>
<td>Proteomics</td>
<td>Human</td>
<td>RPE, choroid, iris-ciliary body</td>
<td>Peptidase proteins detected from the samples</td>
<td></td>
<td>Zhang et al., 2016</td>
</tr>
<tr>
<td>Carboxylesterases</td>
<td>Proteomics</td>
<td>Human</td>
<td>Vitreous, retina</td>
<td>Carboxylesterase proteins detected in vitreous and retina.</td>
<td></td>
<td>Funke et al., 2016; Skeie et al., 2015; Zhang et al., 2015</td>
</tr>
<tr>
<td>Enzyme Class</td>
<td>Substrate and Assay</td>
<td>Species and Tissue</td>
<td>References</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>Substrate hydrolysis in vitro in tissue homogenates and with purified enzymes, immunohistochemistry, proteomics</td>
<td>Human, rabbit, cat, dog, pigeon, rooster, ox, horse, rat, pig</td>
<td>Vitreous, aqueous humor, iris-ciliary body, RPE, choroid (human), iris-ciliary body, retina, aqueous humor, vitreous (rabbit, cat, dog, pigeon, rooster, ox, horse, pig), choroid, optic nerve, conjunctiva (rabbit), neural retina, RPE (rat), vitreous (pig)</td>
<td>Iris-ciliary body and retina had high activities, aqueous humor and vitreous very low (rabbit, cat, dog, pigeon, rooster, ox, horse, pig). Some activity in rabbit choroid, optic nerve and conjunctiva. Acetylcholinesterase contributes less to total esterase activity in rabbit anterior eye than butyrylcholinesterase. A similar trend in rat neural retina and RPE. Differences in contribution between albino and pigmented rabbit and interspecies differences in activity and metabolites. Acetylcholinesterase protein detected in human iris-ciliary body, RPE and choroid. Butyrylcholinesterase activity was detectable in human vitreous and aqueous humor, rabbit cornea, iris-ciliary body and aqueous humor and rat neural retina and RPE.</td>
<td>Appleyard et al., 1991; Ellis, 1971; Koelle &amp; Friedenwald, 1950; Lee et al., 1983; Lee, 1983; Lee et al., 1985; Mains et al., 2012; Petersen et al., 1965; Sánchez-Chávez et al., 1995; Zhang et al., 2016</td>
<td></td>
</tr>
<tr>
<td>Butyrylcholinesterase</td>
<td>Substrate metabolism in vitro with tissue homogenates and purified enzymes</td>
<td>Human, rabbit, rat</td>
<td>Aqueous humor, vitreous (human), conjunctiva, cornea, iris-ciliary body, aqueous humor (rabbit), neural retina, RPE (rat)</td>
<td>Butyrylcholinesterase activity was detectable in human vitreous and aqueous humor, rabbit cornea, iris-ciliary body and aqueous humor and rat neural retina and RPE.</td>
<td>Appleyard et al., 1991; Lee et al., 1983; Lee, 1983; Sánchez-Chávez et al., 1995</td>
<td></td>
</tr>
<tr>
<td>Non-specific esterases</td>
<td>Substrate metabolism in vitro in tissue homogenates, histochemistry, dipivalyl epinephrine metabolism in vivo</td>
<td>Cow, rabbit, human, pig, rat</td>
<td>Corneal epithelium and stroma, iris-ciliary body (cow), aqueous humor, corneal epithelium, stroma-endothelium, iris-ciliary body (rabbit), cornea, conjunctiva, iris (human, pig, rat)</td>
<td>Subcellular esterase activity in the tissues is mostly microsomal and similar to liver and kidney. Esterase compositions in the tissues are different. High activity in corneal and conjunctival epithelia.</td>
<td>Chang &amp; Lee, 1982; Coupland et al., 1994; Lee et al., 1982; Redell et al., 1983</td>
<td></td>
</tr>
<tr>
<td>Paraoxonase 1 (specific)</td>
<td>Aldehyde naphthyl acetate hydrolysis in tissue homogenates in vitro with inhibitors and activity modulators</td>
<td>Rabbit</td>
<td>Cornea, iris-ciliary body, aqueous humor</td>
<td>Paraoxonase activity in cornea, iris-ciliary body and aqueous humor.</td>
<td>Lee et al., 1983; Lee, 1983</td>
<td></td>
</tr>
<tr>
<td>Paraoxonase 1, 2 and 3</td>
<td>Immunohistochemistry</td>
<td>Rat</td>
<td>Whole eyes</td>
<td>Paraoxonase 1 and 3 staining in lens epithelium and fibers, corneal epithelium and retina. Paraoxonase 3 was absent.</td>
<td>Marsillich et al., 2008</td>
<td></td>
</tr>
<tr>
<td>Oxidoreductases</td>
<td>Aldehyde dehydrogenase assay in vitro; proteomics</td>
<td>Human, cow</td>
<td>Cornea (human, cow); aqueous humor, vitreous, retina (human, proteomic studies)</td>
<td>Activity corneal epithelium &gt; stroma &gt; endothelium. Aldehyde dehydrogenase proteins detected in aqueous humor, vitreous and retina.</td>
<td>Boughnelli et al., 2011; Chowdhury et al., 1991; Loukovaara et al., 2015; Mirzaei et al., 2017; Rosenfeld et al., 2015; Skeie et al., 2015; Zhang et al., 2015; Holmes &amp; VandeBerg, 1986</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immunohistochemistry, aldehyde and heptaldehyde metabolism in vitro</td>
<td>Baboon</td>
<td>Cornea, lens, vitreous, retina</td>
<td>Activity in cornea higher than in liver. Low activity in lens and retina.</td>
<td>Boughnelli et al., 2011; Chowdhury et al., 1991; Loukovaara et al., 2015; Mirzaei et al., 2017; Rosenfeld et al., 2015; Skeie et al., 2015; Zhang et al., 2015; Holmes &amp; VandeBerg, 1986</td>
<td></td>
</tr>
</tbody>
</table>

CYP, cytochrome-P450; MAO, monoamine oxidase; RPE, retinal pigment epithelium
### Table 3. Key literature on phase II ocular enzymes.

<table>
<thead>
<tr>
<th>Enzyme family</th>
<th>Method</th>
<th>Species</th>
<th>Tissues</th>
<th>Summary of the results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UDP-glucuronosyltransferases</strong></td>
<td>Substrate metabolism study in tissue homogenates <em>in vitro</em></td>
<td>Rabbit, cow</td>
<td>Cornea, lens, iris, retina, choroid (rabbit), retina, lens, comea, iris, RPE-choroid, ciliary body (cow)</td>
<td>Low activity in rabbit cornea, very low activity in lens, iris, retina and choroid. High activity in bovine ciliary body, low activities in RPE-choroid and iris, very low activities in cornea, lens and retina.</td>
<td>Shichi &amp; Nebert, 1982; Watkins <em>et al.</em>, 1991</td>
</tr>
</tbody>
</table>

RPE, retinal pigment epithelium
2.2 IMPROVING RETINAL DRUG DELIVERY

Intravitreal drug administration is the most efficient way to target the posterior eye segment, and despite its invasiveness, it has been accepted as the gold standard for retinal drug therapy. Novel drug delivery techniques are being explored in attempts to overcome the need for frequent intravitreal drug dosing in the treatment of retinal diseases. Intravitreal drugs can be formulated into solutions, suspensions and various drug delivery devices. From these, drug delivery devices, such as implants, could allow prolonged and controlled drug release, which makes them feasible for chronic drug therapy.

Prodrugs are derived from existing drugs with chemical modification by attaching promoieties, which are cleaved spontaneously or enzymatically. The benefit of the prodrug approach in ocular drug delivery is to optimize a drug’s properties to achieve adequate bioavailability. This can include enhancing solubility or permeability, targeting active transport for increased cell uptake or increasing drug retention time in the vitreous. In the ocular drug delivery field, the traditional rationale behind using prodrugs is enhanced permeability of topical drugs through the cornea. The prodrug approach, however, has also been explored for optimizing drug properties for posterior eye drug delivery.

In the following sections, some of the controlled release drug delivery systems and ocular prodrugs for retinal drug delivery are reviewed.

2.2.1 Controlled drug release systems for intravitreal dosing

Controlled drug release is a desirable property for an intravitreal drug delivery system, since it can extend the duration of action of the drug and consequently decrease the dosing interval. Controlled drug delivery systems can also level out the drug concentration fluctuations in the posterior eye segment, which is important for drugs with a narrow therapeutic index. Intravitreal controlled release drug delivery systems can be formulated either into macroscopic (implants) or particulate (microspheres, nanoparticles) systems from various non-biodegradable and biodegradable polymers (Imperiale et al., 2018). Currently, intravitreal implants are the only controlled drug delivery systems utilized in the clinical treatment of posterior eye segment.

Intravitreal implants can be manufactured from various non-biodegradable and biodegradable polymers (Table 4). Some clinically used intravitreal implants, such as Vitrasert, Retisert and Iluvien, utilize non-biodegradable polymers. The duration of drug release for non-biodegradable implants is long, ranging from six months up to three years, however their large size may require surgical implantation (Christoforidis et al., 2012). Biodegradable polymers undergo hydrolysis, ionization or protonation in the biological fluids (Mitra, 2012). Biodegradable polymers usually have favorable biocompatibility, and good mechanical strength, are small in size, and can be manufactured into various shapes (Christoforidis et al., 2012; Rodrigues da Silva et al., 2010). However, the duration of drug release is in general lower than for
non-biodegradable polymers. The clinically used dexamethasone implant Ozurdex utilizes biodegradable polylactic-co-glycolic acid (Christoforidis et al., 2012).

Table 4. Properties of marketed intravitreal implants.

<table>
<thead>
<tr>
<th>Product</th>
<th>Drug</th>
<th>Implant type</th>
<th>Drug load</th>
<th>Drug release rate</th>
<th>Duration of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitraset*</td>
<td>Ganciclovir</td>
<td>Non-biodegradable</td>
<td>4.5 mg</td>
<td>1.4 μg/h</td>
<td>6-8 months</td>
<td>Chiron, 1997; Dhillon et al., 1998</td>
</tr>
<tr>
<td>Retisert</td>
<td>Fluocinolone acetone</td>
<td>Non-biodegradable</td>
<td>0.59 mg</td>
<td>Initial rate 0.6 μg/day; declines after the first month to 0.3-0.4 μg/day 0.2-0.5 μg/day</td>
<td>2.5 years</td>
<td>Bausch &amp; Lomb Incorporated, 2004; Christoforidis et al., 2012</td>
</tr>
<tr>
<td>Medidur/Iluvien</td>
<td>Fluocinolone acetone</td>
<td>Non-biodegradable</td>
<td>0.19 mg</td>
<td>Most of the dose released during initial 2 months</td>
<td>Up to 3 years</td>
<td>Alimera Sciences Incorporated, 2014; Christoforidis et al., 2012</td>
</tr>
<tr>
<td>Ozurdex</td>
<td>Dexamethasone</td>
<td>Biodegradable</td>
<td>0.7 mg</td>
<td>Most of the dose released during initial 2 months</td>
<td>3-6 months</td>
<td>Allergan Incorporated, 2009; Allergan Pharmaceuticals, 2019; Christoforidis et al., 2012; Lee et al., 2010</td>
</tr>
</tbody>
</table>

* discontinued

The goal of developing controlled release drug delivery systems is to maintain a therapeutic, non-toxic drug concentration in the vitreous for a prolonged time. If drug release from the implant follows zero-order kinetics, e.g. drug is released at a constant rate, then the drug concentration in vitreous will be determined by the rate of the drug release from the drug delivery system and the intravitreal clearance of the drug. For reservoir-type non-biodegradable implants, a constant zero-order drug release rate can be achieved (Rodrigues da Silva et al., 2010). In many biodegradable implant-type drug delivery systems, the drug release follows roughly first-order kinetics: the initial drug release rate slows down with polymer matrix biodegradation (Lee et al., 2010). In this case, the drug concentration in the vitreous is determined by the amount, release rate and intravitreal clearance of the drug. Many biodegradable implants have a burst release of the drug originating from the implant surface and finally during implant disintegration (Christoforidis et al., 2012; Rodrigues da Silva et al., 2010). The drug release rate from implants can be tuned by the choice of polymers and formulation techniques (Imperiale et al., 2018), and experimentally determined in vitro, ex vivo or in vivo.

The drug load in the formulation constrains the concentration that can be reached in the vitreous. In practice, the drug load in the formulation is limited by the loading efficiency, which depends on the drug and its formulation properties (Imperiale et al., 2018), and the maximum formulation volume. In general, 50 µl is
used, since 100-200 µl is considered as the maximum tolerable volume for intravitreal dosing in patients (Kotliar et al., 2007; Pallikaris et al., 2005). The clinically used implants have small volumes, the maximum being 30 µl (Retisert) (Bausch & Lomb Canada Incorporated, 2008), and small drug loads (< 5 mg) (Table 4).

The design of intravitreal drug delivery systems can be aided by pharmacokinetic simulation models that incorporate in vitro and in silico data on the drug and formulation properties. These simulations can also enable the rational selection of formulation for in vivo studies. Pharmacokinetic simulations with varying degrees of complexity have been utilized in the literature to support formulation design. Sarkehl et al. (2014) assessed in vivo drug concentration profiles in the vitreous with drug release data by incorporating the in vitro drug release rates from microspheres into a compartmental model. Del Amo et al. (2015) incorporated in silico predicted intravitreal clearance and volume of distribution into a compartmental model. The model was then used to simulate intravitreal concentration profiles for intravitreal injection and drug delivery systems with various doses, drug release rates and intravitreal drug clearance. This study used a compartmental model, which can be relatively easily built and simulated with integration algorithm software. More complex models, predicting precise concentration gradients in the vitreous, have also been devised to support formulation design. For example, Van Kampen et al. (2018) used finite element modeling to aid the design and fine-tuning of properties (drug release rate, device size) of a hyaluronic acid drug delivery systems.

As reviewed, with intravitreal drug delivery systems, many factors affect the drug concentration in the vitreous. For intravitreal implants, the interplay between clearance, dose, release kinetics and the vitreal concentration has been explored to some extent. However, first-order drug release rates and their interactions with drug dose and drug clearance have not been examined in any detail. Such information could provide valuable insights into formulation design, since biodegradable implants with first-order drug release are a feasible option for extending drug retention in the vitreous. The integration of existing in vivo and in silico data into a compartmental pharmacokinetic model provides an easily accessible tool for exploring the interaction of the parameters.

### 2.2.2 Prodrugs in posterior eye drug delivery

The prodrug approach has been utilized in posterior eye drug therapy for various small molecular drugs, such as antivirals, angiogenesis inhibitors, corticosteroids and antibiotics (Table 5). The design of prodrugs for the posterior eye has aimed at improving intravitreal drug delivery by increasing drug retention in the vitreous and drug exposure in the retina, RPE and choroid (Table 5). Furthermore, some prodrug studies have attempted to reach therapeutic drug concentrations in the posterior eye segment with topical dosing (Doukas et al., 2008; Palanki et al., 2008; Takahashi et al., 2003) or transscleral iontophoresis (Chen & Kalia, 2018; Santer et al., 2018) (Table 5).
Prodrugs have been designed to prolong the retention and duration of effect of intravitreally administered small molecular drugs. One way to achieve this is to use lipophilic prodrug strategies, where drug lipophilicity increases and solubility decreases. Poor solubility enables the formation of crystalline prodrug suspensions in the vitreous, acting as a depot from which the prodrug is slowly solubilized and diffused to the posterior eye tissues. This approach has been utilized for (phosphonomethoxy)ethylguanine (Chen et al., 2017), ganciclovir (Cheng et al., 2002; Cheng et al., 2004), cidofovir (Cheng et al., 2011; Wang et al., 2011), antiproliferative nucleoside analogues (Cheng et al., 2010) and cytarabine (Kim et al., 2012) (Table 5).

Another popular approach has been the design of lipid-conjugated prodrugs that can be incorporated into the walls of liposomes and micelles, or even self-assemble into micelles or liposomes after intravitreal dosing. The lipid formulation allows a sustained prodrug release in the vitreous and consequent prolonged retention of the parent drug. This strategy has been utilized for several antiviral drugs, such as foscarnet (Cheng et al., 1999b; Cheng et al., 1999a), ganciclovir (Cheng et al., 2000), cidofovir (Ma et al., 2015) and acyclovir (Taskintuna et al., 1997). In vivo studies in rabbits, the retention times for the lipid prodrugs have ranged approximately from one (L. Cheng et al., 2000) to two months (Cheng et al., 1999b).

Increasing permeation to retina and RPE with intravitreal dosing can be achieved by lipophilic strategies or targeting prodrugs into active transporters. One group synthetized lipophilic prodrugs of ganciclovir to increase drug permeation to retina and RPE with intravitreal dosing, and to prolong vitreal prodrug retention (Dias et al., 2002; Macha & Mitra, 2002; Majumdar et al., 2004). However, the prodrugs possessed too poor solubilities for further use. The group proceeded into synthetizing peptide prodrugs of ganciclovir, which target the retinal peptide transporters (Patel et al., 2005). With the prodrugs, a slightly higher ganciclovir concentration in the retina was achieved in vivo compared to ganciclovir (Majumdar et al., 2004), though the difference was probably caused by the prodrugs’ higher lipophilicity, not their active transport. A similar approach was used for biotin-conjugated ganciclovir prodrugs, to facilitate their transporter-mediated uptake after intravitreal injection (Janoria et al., 2009). Unfortunately, the conjugation did not result in increased retinal ganciclovir concentrations in vivo. One group synthetized peptide conjugates to facilitate the uptake and controlled bioactivation of a peptide drug in RPE, and found that the cleaving rate could be controlled with the sequence (Bhattacharya et al., 2017).

Lipophilic prodrug strategies and prodrug targeting to transporters have been applied to posterior segment drug therapy via the topical route. In principle, retinal drug therapy with topical dosing seems unfeasible, since the fraction of the drug dose reaching the retina is insignificant. Contradictorily, two lipophilic prodrugs of amfenac and benzotriazine showed efficacy in murine models of choroidal neovascularization with topical dosing (Doukas et al., 2008; Palanki et al., 2008; Takahashi et al., 2003). The finding might be caused by the small size of the murine eye; in fact, there was no evidence of an increased retinal concentration in the rabbit
(Doukas et al., 2008). Transporter-targeted prodrugs have been explored to increase drug concentrations in posterior eye with topical administration via organic cation transporter-, monocarboxylate transporter- and amino acid transporter-mediated uptake (Vooturi et al., 2012), however the increase in vitreal and choroid-RPE concentrations was less than four-fold and probably not clinically relevant.

Table 5. Prodrugs for posterior eye drug delivery divided according to the aim of prodrug design.

<table>
<thead>
<tr>
<th>Compound</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased vitreal retention and slow drug release by decreasing solubility</td>
<td>Chen et al., 2017</td>
</tr>
<tr>
<td>Lipid prodrug of (phosphonomethoxy)ethylguanine</td>
<td>Cheng et al., 2002</td>
</tr>
<tr>
<td>Crystalline lipid prodrug of ganciclovir</td>
<td>Cheng et al., 2011</td>
</tr>
<tr>
<td>Crystalline lipid prodrug of cidofovir</td>
<td>Cheng et al., 2004</td>
</tr>
<tr>
<td>Lipid prodrug of cidofovir and ganciclovir</td>
<td>Cheng et al., 2010</td>
</tr>
<tr>
<td>Lipid prodrugs of antiproliferative nucleoside analogues</td>
<td>Kim et al., 2012</td>
</tr>
<tr>
<td>Lipid prodrugs of cytarabine</td>
<td>Kim et al., 2012</td>
</tr>
<tr>
<td>Increased vitreal retention by lipid conjugation and formation of micelles or liposomes</td>
<td>Taskintuna et al., 1997</td>
</tr>
<tr>
<td>Lipid prodrug of acyclovir</td>
<td>Cheng et al., 1999b; Cheng et al., 1999c</td>
</tr>
<tr>
<td>Lipid prodrug of foscarnet</td>
<td>Cheng et al., 2000</td>
</tr>
<tr>
<td>Lipid prodrug of ganciclovir</td>
<td>Ma et al., 2015</td>
</tr>
<tr>
<td>Increased permeability to posterior tissues by lipophilic prodrug strategies</td>
<td>Chokkar et al., 2014</td>
</tr>
<tr>
<td>Aliphatic ester prodrugs of ganciclovir</td>
<td>Dias et al., 2002</td>
</tr>
<tr>
<td>Increased permeability to posterior tissues by transporter-targeting</td>
<td>Janoria et al., 2009; Majumdar et al., 2004; Patel et al., 2005</td>
</tr>
<tr>
<td>Peptide ester prodrugs of ganciclovir (peptide transporters)</td>
<td>Janoria et al., 2009</td>
</tr>
<tr>
<td>Biotin-conjugated prodrug of ganciclovir (sodium-dependent multivitamin transporter)</td>
<td>Vooturi et al., 2012</td>
</tr>
<tr>
<td>Prodrugs of gatifloxacin (organic cation transporters, monocarboxylate transporters, amino acid transporters)</td>
<td></td>
</tr>
<tr>
<td>Increased corneal permeability by lipophilic strategies</td>
<td>Takahashi et al., 2003</td>
</tr>
<tr>
<td>Nepafenac (amide analogue of amfenac)</td>
<td>Doukas et al., 2008; Palanki et al., 2008</td>
</tr>
<tr>
<td>Ester prodrugs of benzotriazines</td>
<td>Dias et al., 2002; Gao &amp; Mitra, 2000; Macha &amp; Mitra, 2002; Macha et al., 2004</td>
</tr>
<tr>
<td>Aliphatic ester prodrugs of ganciclovir</td>
<td>Chen &amp; Kalia, 2018</td>
</tr>
<tr>
<td>Enhanced iontophoretic permeability by hydrophilic strategies</td>
<td>Santer et al., 2018</td>
</tr>
<tr>
<td>Amino acid ester prodrugs of triamcinolone acetonide</td>
<td>Chen &amp; Kalia, 2018</td>
</tr>
<tr>
<td>Enhanced solubility</td>
<td>Escalona-Benz et al., 2005; Griggs et al., 2002</td>
</tr>
</tbody>
</table>

Most of the previously reviewed prodrugs for posterior eye drug delivery have utilized lipophilic strategies, however hydrophilic strategies might be beneficial in some cases. One example is iontophoresis, where the drug is applied topically and transported through sclera or cornea by application of a mild electrical potential.
Iontophoretic delivery benefits from the drug molecule’s hydrophilicity, good water-solubility and charge, which can be altered by prodrug design. Studies on transscleral iontophoresis of amino acid prodrugs of triamcinolone acetonide (Santer et al., 2018) and acyclovir (Chen & Kalia, 2018) have been conducted *ex vivo* with porcine eye globes. The prodrugs yielded detectable parent drug concentrations in some posterior tissues, such as vitreous, RPE, retina and choroid. The *ex vivo* model lacks choroidal blood flow, which eliminates drugs effectively into the systemic circulation *in vivo*.

The common functional groups in prodrug design in ocular drug development have been carboxylic, hydroxyl, amine and carbonyl groups, which result after modification in ester, carbonate, carbamate, amide, phosphate and oximes (Barot et al., 2012). From these, esterification of carboxylic or hydroxyl groups has become common, since it allows modification of the compound’s lipophilicity as well as achieving sufficient *in vivo* lability (Järvinen & Niemi, 2007). Ester prodrugs can be converted into the active parent drug chemically or enzymatically by ocular esterases, and the bioconversion depends on the acyl and alcohol moieties framing the ester bond: sterically unhindered prodrugs are more rapidly hydrolyzed than the sterically hindered ones (Bundgaard et al., 1988; Chang & Lee, 1982; Chien et al., 1991; Macha et al., 2004). In addition, carbamate and carbonate prodrugs can be synthetized with carboxyl functionalities to increase drug lipophilicity and permeability (Simplício et al., 2008; Zawilska et al., 2013).

The biopharmaceutical requirements for a good prodrug candidate depend largely on the application. A prodrug should have adequate solubility, but also poor water-solubility is beneficial in some cases. The prodrug should also have sufficient chemical stability in the formulation. Moreover, appropriate hydrolysis behavior and bioconversion into the active parent drug in ocular tissues, such as vitreous, retina, RPE and choroid is crucial first for the drug delivery and then for its pharmacological effect. Depending on the application, also permeability and uptake through ocular membranes and cells can be important.

The diversity of biopharmaceutical properties of prodrugs places a methodological challenge for compound screening, which should be conducted for a large number of candidates in a resource-efficient way. The most common early-stage assays usually include metabolic stability and bioconversion studies *in vitro* in various buffers and cell and tissue homogenates, from which the most promising candidates are continued into more complex *in vitro, ex vivo* and *in vivo* studies. In the literature, these fundamental assays are usually conducted with individual prodrugs, necessitating extensive use of time, reagents, tissue material and capacity for analytics. This approach however is suboptimal, and some novel methodological considerations, such as cassette dosing (Pelkonen et al., 2017a; Ramsay et al., 2017; Ramsay et al., 2018), could streamline this property screening.
3 AIMS OF THE STUDY

The aim of this thesis was to contribute to our understanding of the factors affecting ocular drug pharmacokinetics and new strategies and tools for drug delivery to the retina. The specific aims were to:

1. Determine esterase activity in the porcine and rabbit ocular tissues (Pub. I)

2. Develop novel, time- and resource-efficient \textit{in vitro} methods to screen prodrug stability, melanin binding, and hydrolysis and bioconversion in vitreous and RPE (Pub. II)

3. Assess the hydrolytic behavior of various prodrug promoieties in vitreous and RPE \textit{in vitro} (Pub. II)

4. Analyze partition and spatial distribution in the lens, utilizing a novel imaging technique, and explore the effect of drug partition to the lens on pharmacokinetics with topical dosing (Pub. III)

5. Define the effects of drug dose, release rate and clearance on drug concentrations in the vitreous for an intravitreal controlled release drug delivery system (Pub. IV).
4 OVERVIEW OF THE MATERIALS AND METHODS

The materials and methods used for the original publications I-IV are shown briefly in Table 6 and in more detail in the original publications.

Table 6. Overview of the materials and methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Overview</th>
<th>Publications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drug synthesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthesis and characterization</td>
<td>Synthesis of 18 ganciclovir prodrugs</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Nuclear magnetic resonance spectroscopy, mass spectroscopy and elemental analysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vitro methods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ocular tissue collection</td>
<td>Conjunctiva, cornea, aqueous humor, iris-ciliary body, lens, vitreous, neural retina, RPE, choroid and sclera isolation from porcine and/or albino rabbit eyes</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Drug partitioning study</td>
<td>Porcine lenses</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Cassette mix of 16 or 28 small molecular weight drugs, 3 fluorescent dyes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPLC-MS/MS and MALDI IMS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fluorescence microscopy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Image processing</td>
<td></td>
</tr>
<tr>
<td>Esterase activity assay</td>
<td>Porcine and albino rabbit ocular tissues</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Esterase substrate 4-nitrophenyl acetate</td>
<td></td>
</tr>
<tr>
<td>Drug hydrolysis study</td>
<td>Protocol optimization for stability studies</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Stability studies in buffer and porcine vitreous and RPE homogenates</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18 ganciclovir prodrugs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPLC-MS/MS analysis</td>
<td></td>
</tr>
<tr>
<td>Melanin binding</td>
<td>Porcine RPE melanin</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>18 ganciclovir prodrugs</td>
<td></td>
</tr>
<tr>
<td><strong>In silico methods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pharmacokinetic simulations of drug partitioning to ocular lens</td>
<td>Simulations with Stella</td>
<td>III</td>
</tr>
<tr>
<td>In vitro-in vivo scaling of enzyme activity</td>
<td>Tissue protein content and mass</td>
<td>I</td>
</tr>
<tr>
<td>Pharmacokinetic simulations of vitreal drug concentrations with an intravitreal drug delivery system</td>
<td>Scaling by calculations with Excel</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simulations with Stella</td>
<td>IV</td>
</tr>
</tbody>
</table>

HPLC-MS/MS, high performance liquid chromatography tandem mass spectrometry; MALDI IMS, matrix-assisted laser desorption ionization imaging mass spectrometry
5 RESULTS

5.1 ESTERASE ACTIVITY IN OCULAR TISSUES

Esterase activities in various ocular tissues were studied in homogenized porcine and albino rabbit tissues with an esterase substrate 4-nitrophenyl acetate, and the specific activities were scaled to the whole tissue level (Pub. I). The specific activities (substrate hydrolysis rate normalized to sample protein content) were high in conjunctiva, cornea, iris-ciliary body, neural retina, RPE and choroid in both species (Figure 4, panel A). In both pig and rabbit, the lens showed low specific activities.

At the whole tissue level, porcine sclera, neural retina, iris-ciliary body and choroid had high total activities, whereas in albino rabbit, the lens, iris-ciliary body and cornea had high activities (Figure 4, panel B, Table 7). At the whole tissue level, the posterior tissues neural retina and choroid displayed substantial esterase activities. In the pig, neural retina and choroid showed as high an esterase activity as cornea, and in albino rabbit, neural retina exhibited a high activity. In both species RPE possessed low activity. Unfortunately, porcine vitreous showed high non-enzymatic substrate hydrolysis in control experiments, thus the result from the actual experiments was inconclusive. Therefore, data on esterase activity in vitreous is shown only for rabbit.

Comparing the two species, rabbit showed 1.5- to 8-fold higher specific activity for conjunctiva, RPE, iris-ciliary body and lens (Mann Whitney U-test p < 0.01) than the pig, whereas porcine sclera showed slightly (1.3-fold) higher activity than its rabbit counterpart (Mann Whitney U-test p < 0.05) (Figure 4, panel A). At the whole tissue level, the lens and RPE had 10- and 3-fold higher hydrolysis rates in the rabbit than in the pig, respectively (Mann-Whitney p < 0.001) (Figure 4, panel B). Sclera had 10-fold, choroid 8-fold and neural retina 2-fold higher activities in the pig than in the rabbit (Mann-Whitney p < 0.01) (Figure 4, panel B).

Esterase activities in the ocular tissues were also calculated as substrate hydrolysis rates per one gram of tissue to diminish the effect of differences in tissue sizes (Figure 5). Lens and RPE showed 7- and 3-fold higher activities in the rabbit than in the pig, respectively (Mann Whitney U-test p < 0.01). In contrast, choroid showed 2.5-fold higher activity in pig than in rabbit (Mann Whitney U-test p < 0.01).
Figure 4. Esterase substrate 4-nitrophenylacetate hydrolysis in pig and albino rabbit ocular tissues. Specific activities (panel A) were determined with \textit{in vitro} assay and scaled to whole tissues (panel B) with tissue protein content and tissue mass. Comparison to highest activity within species with Dunn’s post hoc test: $p^*<0.05$, $**<0.01$, $***<0.001$. Mean ± SD. (Pub. I)
Table 7. Esterase activities in porcine and albino rabbit ocular tissues calculated as specific activities and scaled to the whole tissue level. Comparison to the highest activity with Dunn’s post hoc test: p ++++ ≥0.05, +++ <0.05, ++ <0.01, +<0.001. (Pub. I)

<table>
<thead>
<tr>
<th>Species and tissue</th>
<th>Specific activity</th>
<th>Activity at the whole tissue level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>++++</td>
<td>NA</td>
</tr>
<tr>
<td>Cornea</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Aqueous humor</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Iris-ciliary body</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Lens</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Neural retina</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>RPE</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Choroid</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Sclera</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Albino rabbit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>++++</td>
<td>NA</td>
</tr>
<tr>
<td>Cornea</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Iris-ciliary body</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Lens</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Vitreous</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Neural retina</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>RPE</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Choroid</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Sclera</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 5. The hydrolysis rate of the esterase substrate, 4-nitrophenyl acetate calculated per gram of ocular tissues for pig and rabbit. Comparison between species with Mann Whitney U-test: *p<0.05, **p<0.01, ***p<0.001. Mean ± SD. (Pub. I)
5.2 IN VITRO SCREENING OF NOVEL GANCICLOVIR PRODRUGS WITH CASSETTE DOSING

The cassette dosing approach was applied to screen chemical stability, hydrolysis in vitreous and RPE, bioconversion in RPE and melanin binding of 18 novel ganciclovir (Figure 6) prodrugs in vitro with respect to intravitreal dosing (Pub. II). The compounds included ganciclovir ester (1, 5-15), carbamate (3, 4) and carbonate (2, 16-18) prodrugs with both mono- and disubstitution and various promoieties. The prodrug bioconversion studies focused on the relevant compounds by first determining the prodrug hydrolysis in RPE homogenate with cassette dosing. Based on the results, only those prodrugs undergoing high hydrolysis proceeded into the bioconversion studies, where each prodrug was incubated individually, and both prodrug and the parent drug were quantified.

Figure 6. Structures of the synthetized prodrugs. (Pub. II)
For vitreous pH buffering, porcine vitreous was mixed with 50 mM Hanks balanced salt solution-Hepes buffer and incubated under 5% carbon dioxide. The system pH was equilibrated to pH 7.5 within one hour and remained at between 7.5 and 7.6 for the whole incubation time (48 h). In unbuffered porcine vitreous homogenate, the initial pH after thawing was 8.3, and it reached pH 8.7 after 24 h incubation in room temperature under atmospheric air.

In the hydrolysis studies in vitreous, three prodrugs were stable (not hydrolysed) (3, 10, 11) (Table 7): these prodrugs had short carbon chains, aromatic rings or branched alkyl chains near the cleaving bond. Four prodrugs (4, 7, 9, 16) showed minor hydrolysis (10-32% hydrolyzed at 35 h) (Table 7). Several prodrugs (1, 2, 5, 8, 12, 13, 15, 17) showed high vitreal hydrolysis (>80% hydrolyzed in 35 h) (Table 7): diesters 8 and 15 and dicarbonate 17 were completely hydrolyzed (>99% at 35 h). Several compounds showed high hydrolysis rates in vitreous i.e. the di- or tri-substituted prodrugs (1, 5, 8, 12, 13, 15, 17).

In RPE homogenate, the hydrolyzed prodrug amount at 2 h ranged from 6.6 to 99.9%. Half of the prodrugs (2, 3, 4, 7, 11, 16) displayed rather low hydrolysis (7-30% at 2 h) (Table 8): Some prodrugs (1, 5, 8, 9, 13, 18) were extensively hydrolyzed (>60% at 2 h) (Table 8): for diester 5 and monoester 9 <1% remained at 2 h. For compounds 8 and 17 calibration curves were not obtained, nonetheless based on the sample responses, the prodrugs were completely hydrolyzed in RPE at 2 h.

In general, based on the decrease of the prodrug concentration at 2 h, it seemed that the prodrugs had higher hydrolysis rates in RPE than in vitreous. In vitreous homogenate, no clear decrease could be seen at 2 h, whereas in RPE, the decrease was substantial for several prodrugs. Prodrug hydrolysis in vitreous and RPE could be attributed to the promoieties used. Carbamate prodrugs (3, 4) and esters with branched alkyl chains (10, 11) were stable in both vitreous and RPE. The prodrugs showing extensive hydrolysis in the tissue homogenates were often disubstituted esters or carbonates with aromatic rings or short alkyl chains. From monoester-diester pairs, the monoesters generally had lower hydrolysis.

The bioconversion into the parent drug, ganciclovir, was studied in RPE with several of the prodrugs (1, 5, 9, 13) by quantitating both prodrug and ganciclovir concentrations. All of the four compounds were bioconverted into ganciclovir, demonstrating their usability as ganciclovir prodrugs (Table 9). With respect to the monosubstituted prodrugs, the prodrug hydrolysis and ganciclovir formation rates matched (Table 9). The prodrug hydrolysis results acquired in the individual incubations matched with those obtained by cassette dosing, supporting the feasibility of cassette approach (Table 8).

With regard to prodrug binding to melanin, all prodrugs except prodrug 12 were low or non-binders (<50% bound). Prodrug 12 with aromatic rings in the two hydroxyl and one amino group showed the highest binding (51±9.8% bound). No high melanin binders (>80% bound) were detected.
Table 8. Prodrug stability in buffer and porcine vitreous and RPE homogenates *in vitro* with cassette dosing. For five prodrugs with adequate stability in buffer and rapid hydrolysis in RPE, bioconversion into ganciclovir was studied in individual incubations. Mean ± SD. (Pub. II)

<table>
<thead>
<tr>
<th>Prodrug no</th>
<th>Stability studies with prodrug mix</th>
<th>Bioconversion study with individual incubations</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS (pH 7.4)</td>
<td>Vitreous</td>
<td>RPE</td>
</tr>
<tr>
<td></td>
<td>Hydrolyzed at 35 h (%)</td>
<td>Hydrolyzed at 35 h (%)</td>
<td>Hydrolyzed at 2 h (%)</td>
</tr>
<tr>
<td>1</td>
<td>stable</td>
<td>83.3 ± 1.8</td>
<td>76.2 ± 3.7</td>
</tr>
<tr>
<td>2</td>
<td>76.4 ± 1.2</td>
<td>96.1 ± 0.2</td>
<td>11.9 ± 3.1</td>
</tr>
<tr>
<td>3</td>
<td>stable</td>
<td>stable</td>
<td>6.6 ± 11.5</td>
</tr>
<tr>
<td>4</td>
<td>stable</td>
<td>31.7 ± 4.8</td>
<td>7.1 ± 4.9</td>
</tr>
<tr>
<td>5</td>
<td>stable</td>
<td>95.1 ± 0.6</td>
<td>99.9 ± 0</td>
</tr>
<tr>
<td>6</td>
<td>stable</td>
<td>NA**</td>
<td>NA**</td>
</tr>
<tr>
<td>7</td>
<td>stable</td>
<td>31.0 ± 4.9</td>
<td>28.5 ± 3</td>
</tr>
<tr>
<td>8</td>
<td>stable</td>
<td>98.9 ± 0.2</td>
<td>NA*</td>
</tr>
<tr>
<td>9</td>
<td>stable</td>
<td>10.2 ± 5.4</td>
<td>94.8 ± 2.5</td>
</tr>
<tr>
<td>10</td>
<td>NA*</td>
<td>stable</td>
<td>NA*</td>
</tr>
<tr>
<td>11</td>
<td>stable</td>
<td>stable</td>
<td>14.2 ± 12.2</td>
</tr>
<tr>
<td>12</td>
<td>NA*</td>
<td>92.7 ± 1.1</td>
<td>NA*</td>
</tr>
<tr>
<td>13</td>
<td>stable</td>
<td>85.6 ± 1.4</td>
<td>76.7 ± 5.1</td>
</tr>
<tr>
<td>14</td>
<td>stable</td>
<td>NA**</td>
<td>NA***</td>
</tr>
<tr>
<td>15</td>
<td>NA*</td>
<td>98.2 ± 0.2</td>
<td>NA*</td>
</tr>
<tr>
<td>16</td>
<td>stable</td>
<td>21.6 ± 7.5</td>
<td>31 ± 7.8</td>
</tr>
<tr>
<td>17</td>
<td>stable</td>
<td>99.1 ± 0.1</td>
<td>NA*</td>
</tr>
<tr>
<td>18</td>
<td>19.2 ± 4.4</td>
<td>NA**</td>
<td>62.9 ± 8.1</td>
</tr>
</tbody>
</table>

NA: result not obtained
*Calibration did not meet acceptance criteria
** Hydrolysis of corresponding tri- or di-substituted prodrug can interfere with the results
*** Depletion of compound for further studies
Table 9. Pseudo-first order reaction rate constants (k) and half-lives (t½) for prodrug hydrolysis and ganciclovir formation in porcine RPE homogenate in vitro. Mean ± SD. (Pub. II)

<table>
<thead>
<tr>
<th>Prodrug no</th>
<th>Di-/monosubstitution</th>
<th>Prodrug hydrolysis</th>
<th>Ganciclovir formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>k (h⁻¹)</td>
<td>t½ (h)</td>
</tr>
<tr>
<td>1</td>
<td>di</td>
<td>0.78 ± 0.05</td>
<td>0.89 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>di</td>
<td>2.28 ± 0.10</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>9</td>
<td>mono</td>
<td>1.73 ± 0.01</td>
<td>0.40 ± 0.003</td>
</tr>
<tr>
<td>13</td>
<td>di</td>
<td>0.43 ± 0.13</td>
<td>1.74 ± 0.50</td>
</tr>
<tr>
<td>18</td>
<td>mono</td>
<td>0.44 ± 0.04</td>
<td>1.58 ± 0.15</td>
</tr>
</tbody>
</table>

5.3 DRUG PARTITIONING AND SPATIAL DISTRIBUTION IN THE LENS

The spatial distribution in the lens (Figure 7) and partitioning between the lens and buffer (Figure 8) were studied in both isolated porcine lenses and cassette mix, and analyzed with MALDI IMS, fluorescence microscopy and LC-MS/MS (Pub. III).

Eleven out of 16 cassette mix compounds displayed acceptable signals in MALDI IMS, when drug standards were pipetted onto blank tissue sections. From the eleven compounds, five drugs (atropine, tizanidine, propranolol, pindolol, and pilocarpine) could be reliably detected from sample lenses (Figure 7). Four compounds showed signals in the sample lenses, but also in blank lenses, thus they were excluded from further analysis.

Based on MALDI IMS data on atropine, pindolol, propranolol, pilocarpine and tizanidine and fluorescence microscopy images on fluorescein sodium, rhodamine-123 and rhodamine-B, the compounds distributed only to the surface layers (capsule and cortex) of the lens after a four-hour exposure (Figure 7). None of the compounds reached the inner lens nucleus. Atropine, pindolol, propranolol, pilocarpine and tizanidine showed 1.2- to 2-fold higher MALDI IMS signal intensities in the posterior rim of the lens compared to the anterior rim.
In general, drug partition to the lens was low, the $K_p$ values ranging from 0.047 to 0.762 (Figure 8). Based on MALDI IMS results, the estimated distribution volume for the compounds was 27.1% of the total anatomical volume of the lens. When this volume of distribution was used in the calculation of $K_p$ values, the $K_p$ ranged from 0.172 (methazolamide) to 2.810 (propranolol) (Figure 8). Even then, only six compounds (propranolol, quinidine, voriconazole, betaxolol, tizanidine, diclofenac) showed $K_p > 1$ (Figure 8), indicating that for most compounds, the drug concentration even in the surface layer of the lens was lower than in buffer. Structurally similar beta-blockers showed a positive correlation between $K_p$ and octanol-water distribution coefficient at pH 7.4 ($\log D_{7.4}$) (Figure 9, panel A). Moreover, an inverse correlation between polar surface area (PSA) and $K_p$ could be seen in the whole group of 28 compounds (Figure 9, panel B).
Figure 9. Correlation between \textit{in vitro} lens-buffer partition coefficient (\(K_p\)) and Log\(D_{7.4}\) (panel A) and PSA of the compounds (panel B). Results for a series of beta-blockers (nadolol, atenolol, carteolol, pindolol, betaxolol and propranolol) shown individually in panel A on the right. \(K_p\) values were calculated by using the true distribution volume (based on imaging) and the total anatomical volume of the lens. (Pub. III)

The pharmacokinetic simulations for topical drug dosing with various lens-buffer partition coefficients revealed that drug partitioning to the lens did not affect drug exposure in the aqueous humor to any significant extent, neither with single (Figure 10, panel A), nor with repeated dosing (Figure 10, panel B). However, the lens-buffer partition coefficient has a substantial impact on lenticular drug exposure, especially with repeated dosing (Figure 10, panel B).

Figure 10. Simulated drug concentration in rabbit aqueous humor and lens with lens-buffer partition coefficients (\(K_p\)) of 0.35 (black line), 1.05 (blue line) and 3.5 (red line) after single (panel A) and multiple (panel B) topical doses. The black symbols in panel A in aqueous humor graph denote \textit{in vivo} data of timolol. For drug concentration in aqueous humor (upper figures), the simulated lines for the three \(K_p\)-values overlap each. (Pub. III)
5.4 PHARMACOKINETIC SIMULATIONS OF INTRAVITREAL CONTROLLED RELEASE DRUG DELIVERY SYSTEM

Intravitreal implants can be divided into non-biodegradable and biodegradable by their degradation behaviour, which also affects their drug release properties: zero-order release can be achieved with non-biodegradable implants (Rodrigues da Silva et al., 2010), whereas drug release from biodegradable implants usually follows first-order kinetics (Lee et al., 2010). The interplay between drug dose, drug release rate and CL_{ivt} and its effect on vitreal drug concentration was explored with pharmacokinetic simulations (Pub. IV). In the simulations, first-order drug release from the drug delivery system was assumed, which resembles drug release from a biodegradable implant. We simulated the vitreal concentration with various drug loads (range from 0.1 µg to 10 mg), drug release rate constants (0.0005-0.0048 h⁻¹, corresponding to t½ of 6-54 days) and CL_{ivt} (0.753 ml/h for fluconazole, 0.04 ml/h for tobramycin, and 0.019 ml/h for bevacizumab), resulting in a total of 135 individual simulations. For the simulations, the drug concentration in vitreous at three months was assessed. Similar explorations have been done previously, however several drug-release rate constants were not used in the simulations (del Amo et al., 2015).

The drug concentrations in the vitreous with various drug load-release rate combinations are presented in Figure 11. Based on the simulations, a lipophilic small molecular weight compound with a high CL_{ivt} (fluconazole) requires a rather high drug loading dose in the device (10 mg) and a slow release rate (t½ > 24 days) if it is to yield vitreal concentrations of 1 µg/ml at 3 months. In contrast, a hydrophilic small molecular weight drug (tobramycin) or macromolecule (bevacizumab) with lower CL_{ivt} can have similar concentrations with ~1 mg drug loads or higher release rates (t½ ≈ 10 days).

The maximum vitreal drug concentrations at 3 months with the highest drug load (10 mg) and slowest drug release (t½ 54 days) depend on the CL_{ivt}. For example, for a lipophilic, highly cleared drug like fluconazole, the maximum concentration at 3 months was < 2.5 µg/ml. With the same drug load and drug release rate, 18-fold higher concentrations could be achieved with tobramycin, which has a low CL_{ivt}. Similar conclusions have been drawn earlier in simulations with amikacin and heptanol (del Amo et al., 2015).
Figure 11. Simulated drug concentration in the rabbit vitreous at three months after intravitreal injection of a controlled release drug delivery system with varying drug release rate constants and drug loads. First order drug release from the system was assumed. Fluconazole, tobramycin and bevacizumab represent lipophilic small molecular weight, hydrophilic small molecular weight and macromolecular drugs, respectively. (Pub. IV)
6 DISCUSSION

6.1 ESTERASE ACTIVITY IN THE EYE

Esterases metabolize several drugs, (e.g. timolol, bimatoprost) and contribute to prodrug bioconversion (e.g. latanoprost, tafluprost, dipivefrin) (Anderson et al., 1980; Chang & Lee, 1982; Chang et al., 1987; Fukano & Kawazu, 2009; Hellberg et al., 2003; Mandell et al., 1978; Redell et al., 1983; Sjöquist et al., 1998), and their activities have been reported in several ocular tissues. In the current work, we studied esterase activities in ten ocular tissues in two species, the pig and the albino rabbit, with a standardized method, which allows a robust comparison of activity between tissues and species. The suggested activity scaling from an in vitro system to the whole tissue level can provide greater insights into the physiological roles of the enzymes. Moreover, as far as we are aware, this is the first time that ocular esterase activities were determined in pig, which is a useful, easily accessible source for animal tissues for both in vitro and ex vivo studies. More information on the activity of esterase subclasses could be obtained by using enzyme-specific substrates or inhibitors instead of the unspecific substrate 4-nitrophenyl acetate (Xie et al., 2002). The results of our work can be used in assessing the metabolism and bioconversion of soft drugs, prodrugs and polymeric drug conjugates.

In the current work, esterase activities were calculated in three different ways: as a specific activity, at the whole tissue level and calculated per gram of tissue. As far as we are aware, these scalars are being presented for ocular tissues for the first time. Specific activities are commonly reported in metabolism studies, and they account for the differences in activity and protein amount in the in vitro assay. They do not however reveal much about the physiological or pharmacokinetic impact of the activity. To achieve this, we scaled the activity to the whole tissue level. In order to evaluate the activities at the whole tissue level, specific activity, protein yield from the tissue and tissue mass must be considered and this may help in the assessment of the total metabolic capacity of a tissue in vivo. We also calculated esterase activities as activity per gram of tissue, which does not take into account the effect of tissue mass. These activities can be useful in interpreting in vitro hydrolysis data, that has not been normalized with the protein amount (such as in Pub. II).

In this study, we found some differences in tissue esterase activities between pig and rabbit. Based on the specific activity, rabbit tissues showed 1.5- to 8-fold higher activities than the pig in conjunctiva, RPE, iris-ciliary body and lens, whereas porcine sclera showed slightly (1.3-fold) higher activity than the rabbit sclera. At the whole tissue level, differences between the species were evident in the lens, RPE, sclera, choroid and neural retina esterase activities. In contrast, differences between the species in esterase activity when estimated per gram of tissue, which considers the specific activity in vitro and the protein yield from tissue, were present only in the lens, RPE and choroid. This indicates that the species differences at the whole
tissue level are caused mostly by species differences in the tissue masses. Our results are in line with the existing literature; however, the method-related differences mean that caution is warranted in the comparison. With respect to the specific activities, similar results have been reported for a comparison between rabbit and porcine cornea and iris-ciliary, i.e. the rabbit tissues having 1.5- to 23-fold higher than their porcine counterparts (Lee et al., 1982). Moreover, esterase activities in cornea, conjunctiva and iris-ciliary body were similar between human, pig and rat (Coupland et al., 1994); unfortunately, the immunohistochemical method did not allow a straightforward comparison of the esterase activities between humans and our present results. Therefore, also the robustness of the albino and porcine rabbit eyes in ocular DME research cannot be discussed based on the similarity with human eye. Albino rabbit is an established animal model in preclinical ocular drug research; thus it may be also a relevant model for studying ocular drug metabolism in vivo. Porcine eyes are readily available from abattoirs and they are relatively inexpensive, which supports their use as a source of tissue material for in vitro assays. In conclusion, both animal models seem to serve a purpose in ocular DME research.

The results of the work can be used when assessing the metabolism of drugs and the bioconversion of ester prodrugs (Pub. II). Esterases can metabolize many drugs and prodrugs, leading to either inactivation or bioconversion. With topical dosing, high drug concentrations are achieved in the corneal epithelium, which also has high esterase activity. The activity in cornea has been used for several clinically used topical prodrugs such as latanoprost, travoprost and dipivefrin (Anderson et al., 1980; Fukano & Kawazu, 2009; Sjöquist et al., 1998). Other tissues that could contribute to the esterase-catalyzed metabolism of topical drugs are aqueous humor, lens and iris-ciliary body. Aqueous humor displays very low activity, and in the lens the activity is likely to be pharmacologically irrelevant, since based on Pub. III, drug concentrations in the lens are low. Iris-ciliary body had high activity; thus, it might contribute to metabolism of topical drugs. For intravitreal drugs, esterase activity at the administration site, in vitreous, is rather low. Neural retina and RPE also possessed some activity, thus they might contribute to the metabolism of some drugs. Based on this work, designing tissue-specific prodrugs cleaved by esterases does not seem feasible, unless the prodrug hydrolysis is known to be esterase subclass-specific.

The current knowledge on ocular enzymes is incomplete. The various methods for studying ocular DMEs all have their advantages and drawbacks, thus they should be used and interpreted in a systematic manner. Furthermore, the role of DMEs in drug and prodrug pharmacokinetics is complex, and cannot be evaluated only with enzyme activity. The process of drug metabolism is dictated not only by the interplay between enzyme activity and enzyme amount, but also by the drug’s tissue permeability, clearance and retention time. This complicates the extrapolation of in vitro results to the in vivo situation. The extrapolation could be improved by integrating various data from in vitro, ex vivo and in silico methods, such as quantitative proteomics, in vitro metabolism and permeability assays and clearances,
into physiological models for in vitro-in vivo extrapolation. Lastly, more information about DMEs in human donor eye tissues is warranted. Extrapolating metabolism from nonclinical animal models to humans needs to be done carefully, since it has been reported that the animal models may be poor surrogates for ocular metabolism in humans (Argikar et al., 2017; Bushee et al., 2015).

6.2 PRODRUG SCREENING WITH CASSETTE DOSING

Cassette dosing has been used previously for studying drug permeability across ocular membranes (Ramsay et al., 2017; Ramsay et al., 2018; Ramsay et al., 2019) and melanin binding (Pelkonen et al., 2017a), as well as ocular drug pharmacokinetics in vivo (Proksch & Ward, 2008). Interestingly, some investigations on in vitro metabolic interaction screening have also utilized n-in-1 dosing (Tolonen et al., 2007; Turpeinen et al., 2005). We believe that this approach has been applied for the first time here in prodrug research. The strategy of studying prodrug hydrolysis in various matrices (buffer, tissue homogenates) as a prodrug mix proved successful. Thus, cassette dosing at low concentrations and HPLC-MS/MS analytics made it possible to assess a large number of prodrugs in a time- and resource-efficient manner. With cassette dosing, special emphasis is placed on HPLC-MS/MS analytics optimization: the structurally similar compounds must be separated adequately within the same sample.

The increase of vitreous pH to >8 after isolation has been reported previously (Patel et al., 2015). A previous publication proposed a method for pH buffering: the system included buffer and vitreous compartments separated by a dialysis membrane (Patel et al., 2015). However, the system is not suitable for small molecular weight drugs, since these compounds would escape through the membrane from the vitreous compartment. Furthermore, the pH equilibration time in the system was rather long, 24 h. In the present work, we validated a simple, fast method to buffer vitreous pH for prodrug hydrolysis studies with buffer addition. Addition of the buffer will, of course, dilute the vitreal enzymes, but we think that the method is reasonable if it is the rank order, not absolute hydrolysis rates, of compounds in the vitreous humor which is desired.

The methods presented in this work can be used for future investigations on ocular prodrugs, as well as soft drugs, e.g. drugs that possess pharmacological activity before metabolism. The cassette dosing approach could also be used for other ocular tissues and also to extraocular tissue matrices.

6.3 EFFECTS OF PROMOIETIES ON PRODRUG HYDROLYSIS IN VITREOUS AND RPE

Information on the impact of promoieties on prodrug hydrolysis in ocular tissues is useful in the design of ocular prodrugs. In the present work, this relationship was
studied with 18 ganciclovir prodrugs with various promoieties in \textit{in vitro} hydrolysis experiments in vitreous and RPE homogenates.

For ester and carbonate prodrugs, hydrolysis in vitreous and RPE increased with carbon chain length. Disubstituted prodrugs also showed extensive hydrolysis in the tissues. Our findings agree with previous reports on alpha- and beta-naphthylesters (Chang & Lee, 1982) and mono- and di-acylated ganciclovir prodrugs (Dias \textit{et al.}, 2002), which indicated that the lipophilic structures led to increased interactions with the hydrophobic enzyme pocket and to subsequent hydrolysis.

Prodrugs with branched carbon chains and aromatic rings close to the ester bond seemed to exhibit elevated stability in vitreous and RPE. A similar finding was observed for timolol ester prodrugs (Bundgaard \textit{et al.}, 1988; Chien \textit{et al.}, 1991). The phenomenon could be due to increased sterical hindrance around the cleaving bond: bulky groups, such as aromatic rings and long, branched carbon chains can hinder enzymatic cleavage. In addition, the carbamate prodrugs showed high stability in tissue homogenates.

Our findings on prodrug hydrolysis in vitreous and RPE homogenates may be attributed to the esterase activities determined in Pub. I. The results on prodrug hydrolysis in vitreous and RPE homogenates are in line with the esterase activities in the rabbit vitreous and RPE; in Pub. I, rabbit RPE showed higher activity than the vitreous in activity when calculated on a per gram-basis. Furthermore, based on esterase activities in the porcine tissues, the prodrugs that are hydrolyzed in the RPE may also be converted to ganciclovir in other ocular tissues, except aqueous humor. Porcine and rabbit tissues showed mostly similar esterase activities on a gram of tissue-basis; thus, the hydrolytic behavior of the prodrugs might be similar in the two species. However, these speculations are largely dependent on the assumption that the prodrugs are hydrolyzed by esterases, which should be clarified by conducting the assays in the presence of enzyme- and esterase subclass-specific inhibitors.

The information on the effect of ester, carbamate and carbonate linkers and the various pro-moieties on the hydrolysis in vitreous and RPE can be used in the design of novel prodrugs for ocular applications. These findings might be applicable also to other ocular tissues, not only vitreous and RPE, but this will need to be confirmed with additional experiments.

\textbf{6.4 LENTICULAR DRUG DISTRIBUTION AND ITS ROLE IN OCULAR PHARMACOKINETICS}

In the current work we studied the lenticular partitioning and spatial distribution of small molecular weight compounds \textit{in vitro} with isolated porcine lenses. We also simulated the impact of lenticular drug partitioning on drug levels in aqueous humor. The number of the studied compounds was extensive, hence our work significantly expands the existing knowledge on drug binding to the lens. Moreover, this was one of the first reports utilizing MALDI IMS to study drug distribution in ocular tissues.
The $K_p$ values for 28 small molecular weight drugs were low, mostly <0.5, and roughly in line with the previous literature on lenticular drug concentrations in vitro and in vivo (Ahmed et al., 1989; Araie et al., 1982; Heyrman et al., 1989; Lee et al., 1991; Ohtori et al., 1991; Tang-Liu et al., 1992). Earlier studies using short incubation times (2-4 h) have yielded $K_p$ values below one (Ahmed et al., 1989; Heyrman et al., 1989), which supports our findings. Some studies report $K_p$ values over ten, when long incubations (e.g. 24 h) have been used (Table 1) (Ohtori et al., 1991; Tang-Liu et al., 1992). However, in our experience, the integrity of the lens is compromised during long incubations; in fact, after 4 h, it was observed that the lens started to visibly swell and break apart, and soon afterwards the lens capsule and cortex came off. Therefore, the long incubations may lead to erroneous estimates of drug partitioning to the lens.

In general, the $K_p$ values support the findings that volume of distribution for intravitreal drugs is close to the anatomical volume of vitreous; this indicates no substantial partitioning to the surrounding tissues, such as the lens (del Amo et al., 2015). For a structurally similar series of beta-blockers, $K_p$ showed that there was a correlation with lipophilicity, which supports the earlier findings on aldose-reductase inhibitors (Ohtori et al., 1991). In the whole data set of 28 drugs, PSA showed an inverse relationship with $K_p$.

We analyzed the spatial distribution of eight compounds in the lens and found that the drugs distributed only to the lenticular capsule, epithelium and cortex. A similar spatial distribution has been reported for timolol (Ahmed et al., 1989), arginine vasopressin and progesterone by mechanical separation of the lens capsule and cortex from the lens nucleus after the incubation (Tang-Liu et al., 1992). The spatial distribution pattern supports the low $K_p$ values obtained with LC-MS/MS analytics. The uneven distribution could be explained by the rather unique biochemistry and anatomy of the lens. The lens capsule and cortex consist of the softer lens fiber cells, which migrate slowly into the posterior lens and accumulate into the lens nucleus. In lens nucleus, the fibers form a tightly packed, high-protein structure with limited extracellular space (Dai & Boulton, 2018). This change in fiber cell organization might be responsible for the gradient in the drug distribution within the lens. Also, in the anterior lens the lens epithelium mat hinder drug diffusion to the lens fiber cells; this was indeed seen as higher MALDI IMS signals in the posterior lens.

In the simulations, high drug partitioning to the lens exerted only an impact on the lenticular drug exposure, leaving the aqueous humor exposure virtually unchanged. The effect was clear even with repeated dosing. The reason for this is presumably the large relative difference between slow intercompartmental clearance between the lens and aqueous humor, and the elimination clearance from aqueous humor to the systemic circulation via aqueous humor outflow. Hypothetically, high drug partitioning to the lens could extend drug exposure in the aqueous or vitreous humors, if adequate time were to be allowed for a drug concentration increase to occur in the lens, e.g. in the case of an extended-release intravitreal implant.
The work has importance in the design of anti-cataract drugs. For nuclear cataract, the drug should be very potent, since presumably only minimal drug concentrations are reached in the lens nucleus. For capsular, subcapsular and cortical cataracts, the requirement for drug potency is not that stringent. Nonetheless, lenticular drug binding does not seem a feasible strategy for increasing drug exposure in the aqueous and vitreous humors.

6.5 IMPACT OF DRUG LOADING, RELEASE RATE AND CLEARANCE IN THE DESIGN OF INTRAVITREAL DRUG DELIVERY SYSTEMS

We simulated an intravitreal controlled release system, such as an implant, with first-order drug release and the simulated vitreal drug concentrations at three months post-injection were obtained. Several parameter values for drug loading, clearance and first-order release rate from the polymer were simulated. We built schematic maps for the design of implants for small molecules (hydrophilic, lipophilic) and macromolecules.

The simulations illustrated that it is drug clearance and potency that mostly dictate how large a leeway is allowed in the implant properties (drug loading capacity, drug release rate). It has been proposed that for high-clearance drugs, high drug loads are needed in order to reach a specific concentration, whereas for low-clearance drugs, much lower loading is sufficient (del Amo et al., 2015). Based on our results, a high drug clearance leads to limitations of drug release rate, if prolonged drug retention in the vitreous is desired. Slower release rates, along with higher drug loads, are required for reaching an adequate vitreous concentration. Obviously, the formulation of drugs benefits if the compounds have high potency, because the target concentrations in those cases are low. In contrast, low clearance drugs can have much higher vitreous concentrations with various release rates and drug loads, making the requirements less stringent (e.g. release rate and drug loading).

The general conclusions from the simulations can be applied in formulation design for small drugs and macromolecules. Moreover, this work exemplifies the use of in silico tools in the design of drug delivery systems. In the model, in silico-predicted or experimentally derived clearance parameters, drug release kinetics, release rate from in vitro or in vivo experiments and drug potency can be integrated to guide the formulation design.
7 CONCLUSIONS

This thesis work provides new insights into factors affecting ocular drug pharmacokinetics and new strategies and tools for drug delivery to the retina. The more specific conclusions are (Figure 12):

1. Specific activities of esterases in the albino rabbit and pig were high in cornea and the iris-ciliary body, and lower in the lens. Rabbit tissues had higher specific activities than their porcine counterparts. A calculation method for scaling ocular enzyme activity from \textit{in vitro} assay to whole tissue level was introduced. The scaled activities were high in cornea and iris-ciliary body and low in the RPE and aqueous humor in both species.

2. Cassette dosing can be utilized for screening the \textit{in vitro} hydrolytic behavior of prodrugs in an efficient manner. A simple and fast method for \textit{in vitro} buffering of the vitreous humor was developed and validated.

3. Promoieties affect prodrug stability in the vitreous and RPE homogenates. Carbamates and prodrugs with bulky functionalities, such as branched carbon chains or aromatic rings, and steric hindrance near the cleaving bond display low hydrolysis rates in the tissues. Prodrugs with long carbon chains at the cleaving bond and disubstitution underwent extensive hydrolysis.

4. Drug partitioning to the lens was low, and the drugs distributed only to the lens capsule and cortex. In topical dosing, lenticular drug partitioning influenced the drug exposure in the lens, but it did not substantially affect drug levels in the surrounding eye tissues.

5. High intravitreal drug clearance imposes significant requirements on the drug loading and drug release from controlled drug delivery systems. For low molecular weight drugs, high potency is required if one wishes to achieve effective long-acting administration with implants and other delivery systems.
Figure 12. Conclusions of the thesis with number referral to the text.

Esterase activity:
- specific activity:
  high in cornea, iris-ciliary body
  low in lens, aqueous humor
  albino rabbit > pig

  activity in tissue level:
  high in cornea, iris-ciliary body
  low in aqueous humor, RPE

1.

In vitro hydrolysis in vitreous and RPE for novel ocular prodrugs:
- n-in-1
- vitreous pH buffering in vitro

2.

Drug distribution to the lens:
- $K_p < 1$
- drugs distribute only to lens capsule and cortex
  - topical dosing:
    high $K_p$ increases drug exposure in the lens, but does not affect the concentration in the aqueous humor

3.

Branched carbon chains/aromatic functionalities, carbamate linkers
  - stability increases
  - Long carbon chains, disubstitution
    - stability decreases

4.

Intravitreal implants:
- High intravitreal CL
  - constrains the drug load and release rate
  - necessitates high drug potency

5.
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Ocular drug development aims to overcome frequent dosing and to innovate new drug therapies. Pharmacokinetic and drug delivery aspects pose a challenge for the development. New information on the factors affecting drug pharmacokinetics in the eye would benefit the development of novel ocular drugs and drug delivery systems. This thesis addresses ocular pharmacokinetics and drug delivery in terms of drug metabolism, drug partitioning, drug delivery system design and method development.