EXPRESSION OF CYTOCHROME P450-ENZYMES AND METABOLISM OF TIMOLOL IN HUMAN OCULAR TISSUE

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Academic dissertation

To be presented with the permission of the Faculty of Medicine, University of Helsinki, for public examination in Lecture Hall 2, Biomedicum Helsinki, Haartmaninkatu 8, on December 18, 2009 at 12 noon.

Helsinki 2009

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ISBN 978-952-92-6475-9 (paperback)
ISBN 978-952-10-5883-7 (PDF)
http://ethesis.helsinki.fi

Yliopistopaino
Helsinki 2009

To my family

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals I to IV and reprinted with the permission of the copyright holders, and on unpublished data:

I Volotinen M, Mäenpää J, Kankuri E, Oksala O, Pelkonen O, Nakajima M, Yokoi T and Hakkola J. Expression of cytochrome P450 (CYP) enzymes in human non-pigmented ciliary epithelial cells; induction of CYP1B1 expression by TCDD. *Invest Ophthalmol Vis Sci* 2009;50:3099-105.

II Volotinen M, Turpeinen M, Tolonen A, Uusitalo J, Mäenpää J and Pelkonen O. Timolol metabolism in human liver microsomes is mediated principally by CYP2D6. *Drug Metab Dispos* 2007;35:1135-41.

III Volotinen M, Korjamo T, Tolonen A, Turpeinen M, Pelkonen O, Hakkola J and Mäenpää J. Effects of selective serotonin reuptake inhibitors on timolol metabolism in human liver microsomes and cryopreserved hepatocytes. *Basic Clin Pharmacol Toxicol* In press.

IV Volotinen M, Mäenpää J, Kautiainen H, Tolonen A, Uusitalo J, Ropo A, Vapaatalo H and Aine E. Ophthalmic timolol in a hydrogel vehicle leads to minor inter-individual variation in timolol concentration in aqueous humor. *Eur J Pharm Sci* 2009;36:292-6.

MAIN ABBREVIATIONS

AHR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
CAI	carbonic anhydrase inhibitor
CAR	constitutive androstane receptor
cDNA	complementary deoxyribonucleic acid
CL_H	hepatic clearance
$CL_{h,int}$	intrinsic *in vitro* clearance
CL_{total}	total systemic clearance
CYP	cytochrome P450
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EM	extensive metabolizer
GR	glucocorticoid receptor
HPLC	high-performance liquid chromatography
IC_{50}	inhibitor concentration producing 50% inhibition of enzyme activity
IM	intermediate metabolizer
IOP	intraocular pressure
K_m	Michaelis-Menten kinetic constant
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NPE	non-pigmented ciliary epithelial
PE	pigmented ciliary epithelial
PM	poor metabolizer
POAG	primary open-angle glaucoma
PXR	pregnane X receptor
RT-PCR	reverse transcriptase-polymerase chain reaction
$t_{1/2}$	half life
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
UM	ultrarapid metabolizer
V_{max}	maximal reaction velocity

ABSTRACT

Timolol has been used for decades to lower increased intraocular pressure (IOP). However, only limited information is available on the metabolism of the drug, especially that in the human eye. The aim of this work was to investigate the expression of drug-metabolizing cytochrome P450 (CYP) enzymes in the ocular tissues, the absorption of timolol into the aqueous humor from two commercially available products, the metabolism of timolol in the human eye and the interaction potential of timolol.

The presence of CYP enzymes in the human ciliary epithelial cells at mRNA level was studied by a reverse transcriptase-polymerase chain reaction (RT-PCR) method. A dose response experiment was carried out for mRNAs of CYP1A1 and CYP1B1 with Northern blotting. The presence of CYP1B1 protein was analyzed by immunoblotting. The metabolism and interactions of timolol were investigated in incubations with human liver homogenate, microsomes and cryopreserved hepatocytes. A clinical trial was undertaken with planned cataract surgery subjects in order to measure timolol concentrations and metabolites of timolol in the aqueous humor.

CYP1A1 and CYP1B1 mRNA were expressed in the human ciliary epithelial cells. CYP1B1 was also expressed at protein level, the expression being strongly induced by the known potent inducer TCDD. CYP1B1 induction is heed to be mediated by aryl hydrocarbon receptor (AHR). Timolol was confirmed to be metabolized mainly by CYP2D6 and potent CYP2D6 inhibitors, especially fluoxetine, paroxetine and quinidine, inhibited its metabolism. This inhibition may be of clinical significance in patients using ophthalmic timolol products. Low levels of CYP2D6 mRNA splice variants were expressed in the human ciliary epithelial cells and very low levels of timolol metabolites were detected in the human aqueous humor. A negligible amount of CYP2D6 protein is evidently expressed in the human ocular tissues. Timolol 0.1% eye gel leads to an aqueous humor concentration high enough to achieve a therapeutic effect. Inter-individual variation in concentrations is low and systemic safety can be

increased by using the product with a lower timolol concentration instead of 0.5% eye drops.

1 INTRODUCTION

The common eye disease glaucoma can cause irreversible blindness if not diagnosed and treated in the early stages of progression (see Lee and Higginbotham 2005). This disease is often, albeit not always, associated with increased intraocular pressure (IOP), which is also the most important risk factor for glaucoma (Fuchsjager-Mayrl et al. 2005). Treatment focuses on reduction of IOP, which is nowadays the only treatment option. Reduction may be achieved either pharmacologically, with laser or surgically. Medical treatment involves topical β-adrenergic antagonists, α-adrenergic agonists, miotics, prostaglandin analogs, and oral and local carbonic anhydrase inhibitors (CAIs) (Langham 1971, Hoyng and van Beek 2000).

Ophthalmologically timolol has been used for the treatment of glaucoma and increased IOP for thirty years. Conventional timolol eye drops are available in concentrations of 0.25% and 0.5%. In order to improve safety an 0.1% timolol eye gel has been developed. Timolol is locally fairly well tolerated but may cause e.g. adverse cardiovascular effects due to systemic absorption. It has been reported that approximately 80% of a topically administered eye drop is systemically absorbed (Shell 1982, Korte et al. 2002).

After absorption the drug is distributed, metabolized and eliminated from the body. For elimination a drug often needs to be transformed into a more polar water-soluble and excretable metabolite (Meyer 1996). The major organ for this biotransformation is the liver, but other organs can also contribute to the process. Two metabolic routes, phase I and phase II reactions, are responsible for the transformation of the majority of drugs (Wijnen et al. 2007). Phase I reactions are induced mainly by cytochrome P450 enzymes and involve hydroxylation, reduction and oxidation of substrates.

Cytochrome P450 (CYP) enzymes are a superfamily of heme-containing enzymes (see Conney 1982, Guengerich 1992, Sikka et al. 2005). Each P450 enzyme is encoded by a different gene. Enzymes are divided into families, subfamilies and isoforms based on amino acid sequence identity (Attar et al. 2005, Sikka et al. 2005, Wijnen et al. 2007).

The metabolism of timolol has not been extensively studied even though the drug has been used for decades. It has been suggested that timolol is metabolized by cytochrome P450 enzyme CYP2D6 (Edeki et al. 1995, Ishii et al. 2000).

The aim of this work was to investigate the metabolism and interactions of ophthalmic timolol, focussing on the expression of drug-metabolizing CYP enzymes in human ciliary epithelial cells *in vitro* and the metabolism of timolol in a clinical study in the human. The effects of timolol eye gel and eye drop formulations on timolol pharmacokinetics were also investigated.

2 REVIEW OF THE LITERATURE

2.1 Eye and intraocular pressure

The health of the human eye is dependent on the continuous supply of aqueous humor which circulates through its chambers (for a review see Brubaker 1991). The aqueous humor flows from the posterior chamber to the anterior chamber of the eye (see Figure 1 for the anatomy of the eye). The aqueous humor has two main functions: to nourish avascular structures such as cornea and lens and to maintain IOP levels (for a review see Ferrer 2006). Unchanging IOP maintains the curvature of the cornea, which maintains the refractory properties of the eye (for a review see Civan and Macknight 2004).

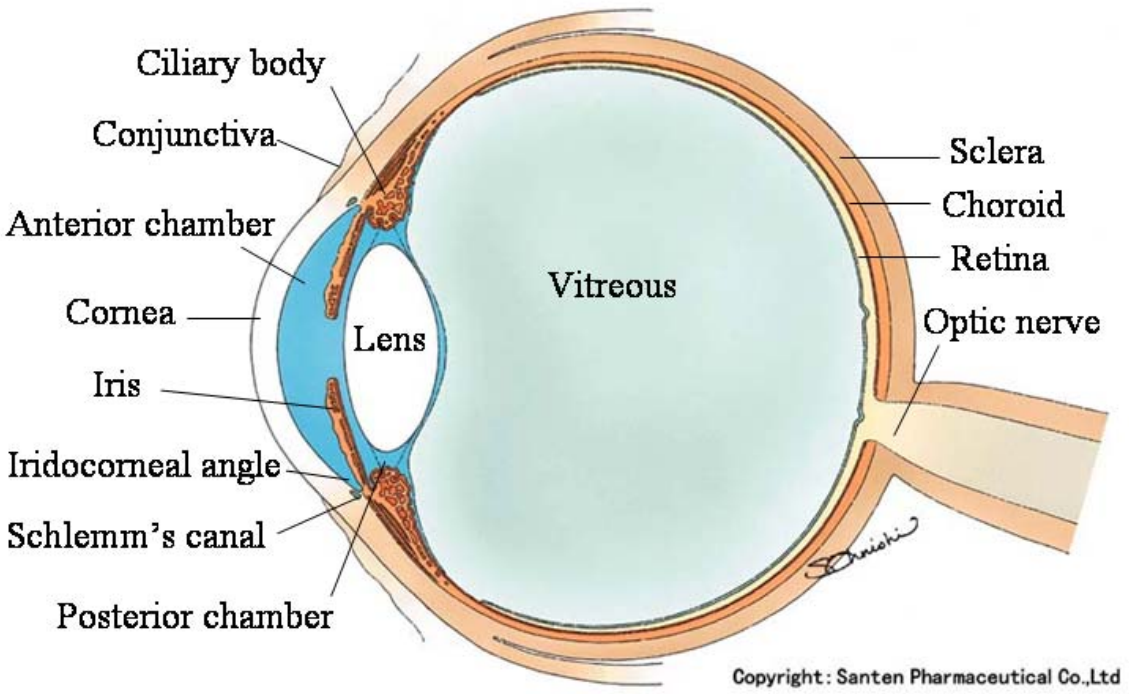

Figure 1. Anatomy of the eye.

Intraocular pressure is formed as a balance between aqueous humor production/ secretion and outflow (Brubaker 2003). The aqueous humor is formed in the ciliary body by the ciliary epithelium, which comprises pigmented ciliary epithelial (PE) and non-pigmented ciliary epithelial (NPE) cells (Ferrer 2006, Coca-Prados and Escribano 2007). The pigmented ciliary epithelial cell layer faces the stroma, the non-pigmented layer the posterior chamber of the eye (Civan and Macknight 2004). The cells in these layers are linked by gap junctions. Aqueous humor is secreted across the ciliary epitheliun as a result of an energy-dependent active transport of solute, mainly NaCl, from the stroma to the posterior chamber of the eye, with water passively following. The secretion process can be divided into three steps: uptake of NaCl from stroma to PE cells by electroneural transporters; passage of NaCl through gap junctions from PE to NPE cells and finally release of Na^+ through Na^+, K^+ -activated ATPase and Cl^- through Cl^- channels. The composition of the aqueous humor differs from that of plasma mostly in protein and ascorbate concentrations (Gabelt and Kaufman 2003). The protein concentration is low, 200 times less than in plasma, while the ascorbate concentration is high, 20 times greater than in plasma. After secretion from the ciliary epithelial cells the aqueous humor passes from the posterior chamber to the anterior chamber, where lie the places of outflow (Figure 2) (Ferrer 2006, Coca-Prados and Escribano 2007). The main outflow route is the trabecular route, through the trabecular meshwork and Schlemm's canal. The other, the uveoscleral route plays a smaller role. The rate of aqueous humour flow is 2 - 3 µl/min in healthy subjects (Johnson and Kamm 1983, Brubaker 1991). The rate has been reported to be associated with circadian variation, evincing a higher rate of secretion during the day and lower at night, the rate of secretion falling 50-60% during sleep (Gherghel et al. 2004, Coca-Prados and Escribano 2007).

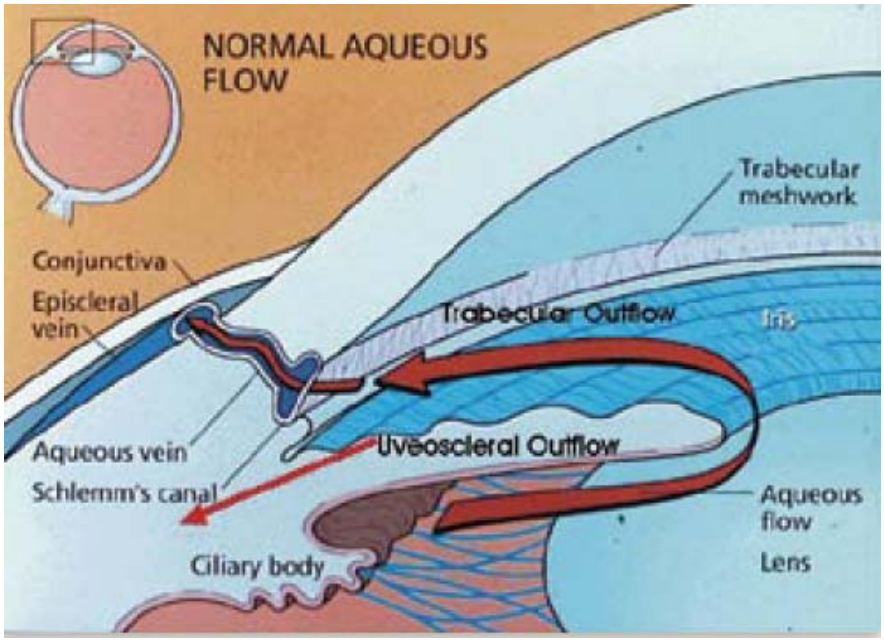

Figure 2. Aqueous humor flow in the eye (Adatia and Damji 2005).

In a general population, IOP ranges between 11-21 mmHg, the average being approximately 16 mmHg (Kallaur and Fingeret 2001, Kanski 2003). There is no absolute cut-off value for increased intraocular pressure; however 21 mmHg is considered a kind of upper limit for normal pressure. IOP is known to fluctuate throughout the day and night, the mean diurnal variation being 5 mmHg in normal eyes. Kida and associates (2006) studied 24-hour changes in IOP in healthy young volunteers and found the nocturnal mean IOP to be significantly higher than the diurnal (see Figure 3). Hara and co-workers (2006) reported similar findings among untreated primary open-angle glaucoma patients. In patients with IOPs less than 21 mmHg during clinic hours, 20% had a reproduced IOP of 21 mmHg or greater while asleep.

In sitting or standing position the eye is approximately 30 cm higher than the heart (Hara et al. 2006), while supine the eye and the heart are at almost the same level. Episcleral venous blood flow is thought to be affected by changes in position, one conception being that the increase in resistance in aqueous flow is a reason why the IOP is higher in supine as compared to sitting position. Understanding the diurnal variation in IOP may have an essential role in better understanding the relationship between IOP

and the optic neuropathy (Weinreb and Liu 2006). Especially when considering patients with glaucomatous changes but well-controlled IOP values during the diurnal/awake period in office hours, the possibility of elevated IOP during sleep should be considered.

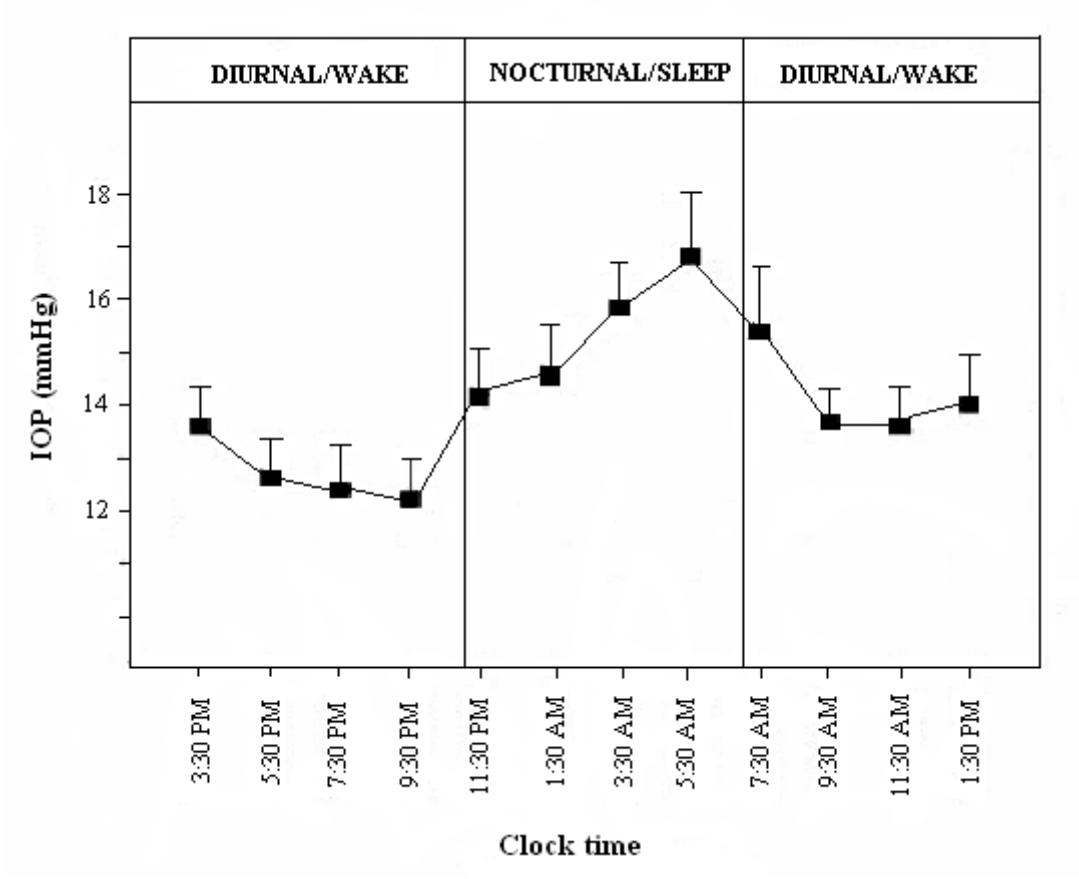

Figure 3. The 24-hour IOP variation in healthy young adults (modified from Kida et al. 2006).

2.2 Glaucoma

Glaucoma is a common eye disease which can cause irreversible blindness if not diagnosed and treated (Lee and Higginbotham 2005). It is the second leading cause of irreversible blindness worldwide (Weinreb and Khaw 2004, Nduaguba and Lee 2006). Glaucoma actually comprises a group of progressive optic neuropathies which have in common damage to the optic nerve and a distinct appearance of the optic disc

manifested clinically as cupping of the disc (Brooks and Gillies 1992, Weinreb and Khaw 2004). This cupping is a sign of glaucomatous optic neuropathy. There are approximately 1.2 million ganglion cells in the optic nerve head and the bodies of these cells are located in the retina (Adati and Damji 2005). The decrease in the number of these retinal ganglial cells in a glaucoma patient leads to neural rim thinning and enlargement of the optic nerve cup (Distelhorst and Hughes 2003), this nerve fiber loss causing a permanently decreased visual field. Though the pathogenesis of glaucomatous optic neuropathy is not totally understood, it has been suggested that ocular blood flow and the metabolism of oxygen causing oxidative stress are involved (Mozaffarieh et al. 2008).

There are several different types of glaucoma, classically divided into primary and secondary open-angle or angle-closure glaucoma (Lee and Higginbotham 2005): see Table 1 for a clinical classification of glaucomas. Glaucoma is considered primary when no specific cause can be identified, and secondary when optic neuropathy follows a high rise in IOP resulting from some condition in the eye such as inflammation, trauma or abnormal pigment in the anterior chamber (Brooks and Gillies 1992).

Table 1. Clinical classification of glaucoma (Kallaur and Fingeret 2001).

Primary glaucomas
Open-angle glaucomas
Chronic open-angle glaucoma
Normal tension glaucoma
Angle-closure glaucomas
Acute angle-closure glaucoma
Subacute angle-closure glaucoma
Chronic angle-closure glaucoma
Secondary glaucomas
Secondary open-angle glaucomas
Pigmentary glaucoma
Exfoliation glaucoma
Steroid-induced glaucoma
Uveitic glaucoma
Secondary angle-closure glaucomas
Neovascular glaucoma
Posttraumatic glaucoma
Congenital glaucoma

2.2.1 Prevalence of glaucoma

The prevalence of glaucoma is estimated to be almost 70 million people worldwide, with at least 6.8 million bilaterally blind. The prevalance is estimated to be on the increase and the condition is projected to affect 79.6 million people by 2020 (Gupta and Yülel 2007). Primary open-angle glaucoma is perhaps the most common type, especially in European and African populations (Weinreb and Khaw 2004). It is estimated that in the developed countries less than 50% of glaucoma patients are aware of having this sight-threatening disease and in the developing countries the proportion of undetected cases is even higher (Quigley 1996). The prevalence of the disease is markedly age-dependent, being approximately 1-2% in persons over the age of 40 and increasing with age (Novack 2003). Over 90% of all patients with glaucoma are above the age of 55 years (Nduaguba and Lee 2006). In different studies the prevalence of

open-angle glaucoma has been reported to be from 1.5% to 5.7% in persons 65-74 years of age (Ekström 1996).

2.2.2 Risk factors for glaucoma

Glaucoma may occur in some individuals with an IOP within the normal range (Brooks and Gillies 1992). However, the risk of glaucomatous damage increases with higher IOP values. A patient with an IOP over 24 mmHg has a 10.5 times higher risk of glaucomatous changes as compared to a patient with an IOP of less than 16 mmHg.

A family history increases the risk almost tenfold (Nduaguba and Lee 2006). A parent or sibling of a known glaucoma patient has an increase of 22% in lifetime risk of developing open-angle glaucoma compared with the general population. This suggests that there is a genetic component associated with open-angle glaucoma development. Several other forms of glaucoma have also been linked with specific genes and gene logi (Cohen and Allingham 2004). However, the genetic background of the condition is complex and results from interactions of multiple genetic factors, and is also suspectible to the influence of environmental factors (Wiggs 2007). Mutations in the myocilin gene have been associated with early-onset as well as adult-onset open-angle glaucoma. Optineurin is another gene mutations in which are associated with open-angle glaucoma. Normal tension glaucoma has also been associated with mutations in this gene. Optineurin is proposed to participate in the tissue necrosis factor α signaling pathway, one pathway thought to be involved in retinal ganglion cell apoptosis in normal-tension and POAG patients. A third gene associated with glaucoma and specifically with pseudoexfoliation syndrome, a strong risk factor for glaucoma, is the lysyl oxidase-like 1 gene (LOXL1) (Aragon-Martin et al. 2008, Lemmelä et al. 2009, Challa 2009). These genes, as well as several others suggested to be associated with some forms of glaucoma, have been found to be responsible for only a small fraction of glaucoma cases.

Glaucoma has been reported more often in non-Caucasian races (Sommer et al. 1991). Leske and associates (2007) reported higher incidences of open-angle glaucoma in

subjects of African origin compared with overall glaucoma incidences. In this population the incidence increased with age, being 2.2% at ages 40 to 49 and 7.9% at ages 70 years or older. It tended to be higher in men than in women. Rudnicka and co-workers (2006) reviewed published open-angle glaucoma prevalence studies and obtained similar results: black populations had the highest open-angle glaucoma prevalence at all ages. However, the increase in prevalence with age was highest in Caucasian populations. Also according to the analysis in question men were more likely than women to have glaucoma.

Other risk factors are also frequently mentioned. However, the evidence of their existence is not strong or is contradictory. Diabetes and hypertension have been suggested as risk factors for glaucoma (Kroese and Burton 2003, Tarkkanen 2008). In epidemiologic studies positive, negative and no significant relationship have been reported for hypertension (Leske et al. 2008). In some studies young hypertensives were less likely to have open-angle glaucoma and older were more likely to have it (Boland and Quigley 2007). It has also been reported that increased blood pressure and hypertension are associated with IOP and high-tension glaucoma but not with normal tension glaucoma (Dielemans et al. 1995). Type 2 diabetes and elevated serum glucose levels have been associated with increased IOP and open-angle glaucoma in some studies (Dielemans et al. 1996; Chopra et al. 2008).

Certain structural differences in the eye have also been suggested as risk factors. Myopia has been associated with open-angle glaucoma and hypertropic eyes with angle closure glaucoma (Kallaur and Fingeret 2001, Boland and Quigley 2007). The association between central corneal thickness and glaucoma has been studied and it has been found that thinner central corneal thickness is related to open-angle glaucoma and stage of glaucoma damage (Congdon et al. 2006, Boland and Quigley 2007, Leske et al. 2008). The explanation for this association is not clear. It is known that thinner corneas give lower values in IOP measurement. This may cause glaucoma subjects with IOP measurements to go undetected or lead to less aggressive IOP-lowering therapy in such patients. It has also been suggested that a thinner cornea could be an indicator of

biomechanical and structural characteristics of ocular tissues which may have an effect on open-angle glaucoma risk.

Drugs used for the treatment of other diseases, systemic or ophthalmic, may have a role in the development of glaucoma. Steroid use is known to cause an increase in IOP (Boland and Quigley 2007). Long-term use of ophthalmic corticosteroids as well as oral intake of large doses has been associated with open-angle glaucoma. Recently, also steroid-containing nasal sprays and inhaled medications have been suggested to increase the risk of glaucoma (Boland and Quigley 2007).

2.2.3 Types of glaucoma

2.2.3.1 Primary open-angle glaucoma

Primary open-angle glaucoma (POAG) is the most common type of glaucoma in several populations (Novack 2003, Leske et al. 2007). POAG is a chronic disease which is generally bilateral but can also occur asymmetrically (Weinreb and Khaw 2004). This disease form has an adult onset and patients present with an anterior chamber of normal appearance. Chronic open-angle glaucoma is difficult to identify due to the absence of symptoms at the time of diagnosis and therefore it is underdiagnosed (Adatia and Damji 2005). It has been suggested that half of glaucoma cases go undetected and therefore untreated.

The pathophysiology of open-angle glaucoma includes a progressive decrease in the number of retinal ganglial cells (Distelhorst and Hughes 2003). This leads to neural rim thinning and enlargement of the optic nerve cup. A consequence of this nerve fiber loss is a permanently decreased visual field. Diagnostic findings in open-angle glaucoma are a symmetrically enlarged cup-to-disc ratio (>5), an asymmetry of two or more in the ratio between the eyes or a highly asymmetric cup in one eye. Even though the reason for decreased vision caused by glaucoma lies in the optic nerve head, the only risk factor amenable to treatment so far is intraocular pressure. In POAG the aqueous outflow is impaired due to dysfunction of the drainage system: aqueous outflow by the

trabecular meshwork and uveoscleral routes is diminished (Figure 4) and can be treated with drugs affecting this system.

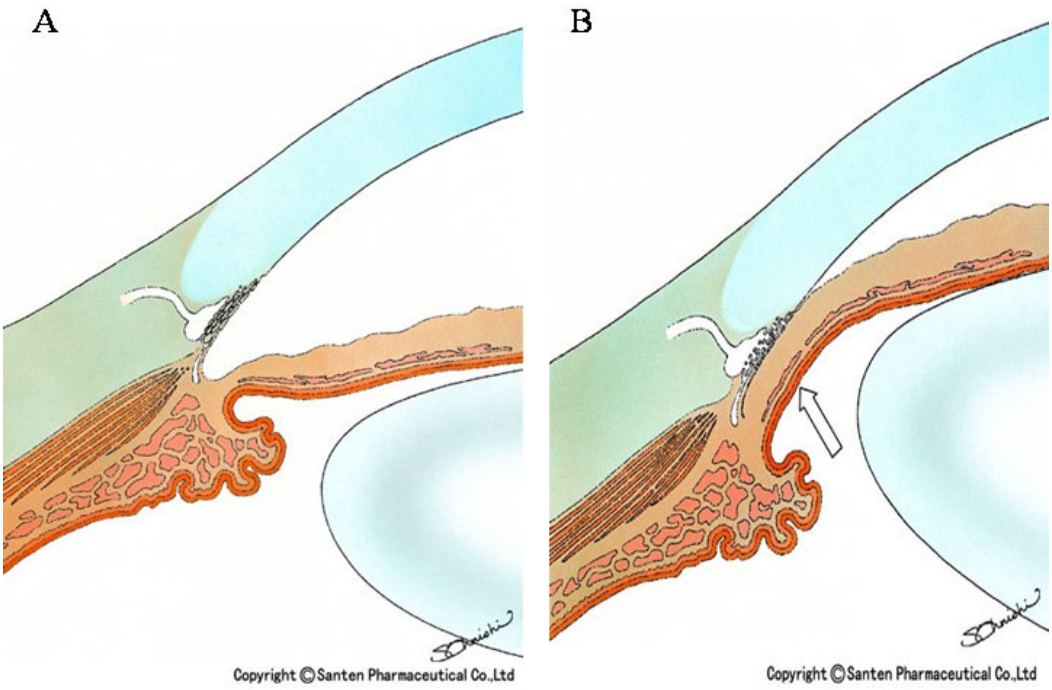

Figure 4. In open-angle glaucoma (A) the iriodocorneal angle is open and in angle-closure glaucoma (B) the iridocorneal angle is closed due to the abnormally positioned iris.

2.2.3.2 Normal tension glaucoma

In normal tension or low tension glaucoma, as it is also termed, patients have typical glaucomatous optic nerve degeneration even though their IOPs are not elevated (Wiggs 2007). Normal tension glaucoma is a form of primary open-angle glaucoma, 25-50% of POAG patients having normal IOP and classified as normal tension glaucoma (Kroese and Burton 2003). Even though these patients show normal IOP, the treatment of the disease is the same as for POAG patients, reduction of IOP. It has been shown that IOP reduction is beneficial in disease progression also in this glaucoma form.

2.2.3.3 Angle-closure glaucoma

Angle-closure glaucoma can be either acute or chronic (Brooks and Gillies 1992). Acute angle-closure glaucoma is the most common form of the acute disease. Rapid onset and a high rise in IOP are characteristic of this glaucoma form. In angle-closure glaucoma impaired aqueous outflow results from anterior chamber occlusion due to an abnormally positioned iris, which blocks aqueous outflow through the iridocorneal angle (Figure 4) (Distelhorst and Hughes 2003). This causes a rapid rise in IOP. In addition to increased IOP signs of acute angle closure include conjunctival injection, corneal epithelial edema, mid-dilated unreactive pupil and in the presence of an occludable angle a shallow anterior chamber (Saw et al. 2003). Symptoms are ocular pain, headache, nausea, vomiting, malaise and a history of intermittent blurring of vision with haloes. Angle-closure glaucoma is defined when the patient has at least three of the symptoms and at least two of the signs. Acutely increased IOP should be reduced. Generally treatment is laser iridotomy or iridectomy (Distelhorst and Hughes 2003). Medical therapy can be used as additional treatment, but is not a substitute for laseration.

2.2.3.4 Secondary glaucomas

Secondary glaucoma develops as a result of another recognizable disease process (Kallaur and Fingeret 2001). Typical glaucomatous signs of open-angle or angle-closure glaucoma are usually present in patients with secondary forms of these diseases. Secondary open-angle glaucoma can be caused by many conditions and the glaucomas in question are named according to the cause. Pigmentary glaucoma is associated with the pigmentary dispersion syndrome, in which pigment lost from the posterior surface of the iris is dispersed into the anterior chamber. Exfoliation glaucoma is associated with the exfoliation syndrome, in which aqueous outflow is decreased due to exfoliated material in the trabecular meshwork. Steroid-induced glaucoma is a consequence of long-term steroid use. Anterior uveitis can cause changes in the anterior chamber which reduce aqueous outflow and increase IOP, leading to uveitic glaucoma. Neovascular glaucoma is a secondary angle-closure glaucoma which results from growth of new vessels in the iris. Posttraumatic glaucoma can be formed after blunt trauma to the eye causing damage to the anterior chamber angle and reduction in aqueous outflow.

2.2.3.5 Congenital glaucoma

Congenital glaucoma, also known as infantile glaucoma, is a rare form of glaucoma. This glaucoma form is a result of fetal maldevelopment of the trabecular meshwork and anterior chamber angle which leads to obstruction of aqueous outflow, increased IOP and optic nerve damage (de Luise and Anderson 1983). The symptoms of the disease are tearing, photophobia, corneal edema, corneal clouding and buphthalmos (enlargemant of the globe) (Hollander et al. 2006, Kumar et al. 2007). Congenital glaucoma usually manifests within the first year of life, but can appear up to the age of three years (Sarfarazi 1997). The indidence of the disease ranges from 1:1 250 to 1:22 000 births in different populations (Messina-Baas et al. 2007). Medical therapy has only a supportive role in the treatment of congenital glaucoma, the primary treatment being surgical (de Luise and Anderson 1983).

2.2.3.5.1 Association between congenital glaucoma and *CYP1B1* gene

Most congenital glaucoma cases are sporadic; however, analysis of affected families supports the conception of an autosomal recessive inheritance (WuDunn 2002). In the literature congenital glaucoma has been associated with chromosomal abnormalities in at least 17 different autosomes (Sarfarazi 1997). Especially homozygous mutations in the *CYP1B1* gene, which codes cytochrome P450 enzyme 1B1 and is located on chromosome 2p22-p21 has been linked to congenital glaucoma (Messina-Baas et al. 2007, Vasiliou and Gonzalez 2008). Though the role of CYP1B1 in the developing eye is not known (WuDunn 2002), it has been suggested that it participates in iridocorneal angle development (Lopez-Garrido et al. 2006). In the eye, *CYP1B1* has been reported also to be expressed in the iris (Coca-Prados and Escribano 2007). *CYP1B1* gene mutations have been identified in affected families from various Western and Middle Eastern regions (WuDunn 2002). Recently, *CYP1B1* mutations have been identified in Japanese congenital glaucoma patients. Sequence alterations in *CYP1B1* gene have been indentified in 4-9% of patients from France, India and Spain (Coca-Prados and Escribano 2007).

2.2.4 Treatment of glaucoma

The treatment of glaucoma is focused on the reduction of IOP, which is the most important identified risk factor for the disease (Fuchsjager-Mayrl et al. 2005). IOP reduction may be achieved either pharmacologically or surgically. Laser treatment: laser trabeculoplasty and laser diode cyclophotocoagulation or incisional surgery: trabeculectomy and deep sclerectomy are methods which can be used to lower IOP for patients who do not respond to antiglaucoma medications (Weinreb and Khaw 2004, Lee and Higginbotham 2005). In cases of congenital glaucoma surgical therapy is the primary treatment.

2.2.4.1 Medical treatment

The medical treatment of glaucoma has long comprised of topical β-adrenergic antagonists, α-adrenergic agonists, miotics such as cholinergic agonists, and oral carbonic anhydrase inhibitors (CAIs) (Table 2) (Langham 1971, Hoyng and van Beek 2000). The newest classes of topical agents are prostaglandin analogs and local CAIs. Treatment is usually initiated with a topical selective or nonselective β-adrenergic antagonist or a topical prostaglandin analog (Lee and Higginbotham 2005). Second-choice drugs include α-adrenergic agonists and topical CAIs. Cholinergic agonists, e.g. pilocarpine, are third-line options. Oral CAIs such as acetazolamide may be used temporarily in patients with elevated IOP, e.g. postsurgery or continuously in patients with glaucoma not responding to other treatment (Hoyng and van Beek 2000). Combination therapy may be used when the target pressure for a patient cannot be reached with monotherapy.

The target in glaucoma treatment is to achieve an IOP level low enough to stop the progression of glaucomatous damage (Cohen et al. 2004). Slightly different recommendations for IOP reduction have been published. IOP lowering by at least 20% has been proposed in moderate glaucoma and 40% to 50% in severe glaucoma (Schwartz and Budenz 2004). Also a 20% reduction from the initial intraocular pressure or a decrease to < 18 mmHg in advanced glaucoma and a 25% reduction in patients with inital glaucoma has been recognized as a favorable strategy to reach target

intraocular pressure (Popović-Suić et al. 2005). However, the target figure for IOP in each patient depends on the pretreatment pressure level, optic nerve condition, glaucoma disease state, rate of glaucoma progression, patient's age, and other risk factors for the development of the disorder. The Finnish Current Care guideline for glaucoma recommends a reduction of at least 25% and the target IOP level may be lower e.g. in patients with several risk factors (Finnish Medical Society Duodecim 2007).

Table 2. Drugs used for the treatment of glaucoma in Europe.

Drug action/class	**Agent**	**Concentration and formulation**	**Frequency of administration**
Drugs which reduce aqueous production			
β-Adrenergic antagonists	Betaxolol	0.5 % eye drops	Twice a day
	Carteolol	1 % eye drops	Twice a day
	Levobunolol	0.25 % eye drops	Twice a day
	Timolol	0.25 and 0.5 % eye drops	Twice a day
		0.1 and 0.5 % eye gel	Once a day
α-Adrenergic agonists	Apraclonidine	0.5 and 1 % eye drops	Three times a day
	Brimonidine	0.15 and 0.2 % eye drops	Three times a day
Carbonic anhydrase inhibitors	Acetazolamide	125, 250 and 500 mg tablets	2 - 4 times a day
	Brinzolamide	1 % eye drops	Three times a day
	Dorzolamide	2 % eye drops	Three times a day
Drugs which increase aqueous outflow			
Cholinergic agonists (miotics)	Carbachol	0.75 to 3 % eye drops	3 - 4 times a day
	Pilocarpine	1 and 2 % eye drops	3 - 4 times a day
Prostaglandin analogs	Bimatoprost	0.03 % eye drops	Once a day
	Latanoprost	0.005 % eye drops	Once a day
	Tafluprost	0.0015 % eye drops	Once a day
	Travoprost	0.004 % eye drops	Once a day
	Unoprostone	0.15 % eye drops	Twice a day
Non-selective adrenergic agonists	Dipivefrin	0.1 % eye drops	Twice a day
Combination products			
	Timolol + bimatoprost	0.5/0.03% eye drops	Once a day
	Timolol + dorzolamide	0.5/2 % eye drops	Twice a day
	Timolol + latanoprost	0.5/0.05 % eye drops	Once a day
	Timolol + pilocarpine	0.5/2 and 0.5/4 % eye drops	Twice a day
	Timolol + travoprost	0.5/0.004% eye drops	Once a day

References: Bartlett et al. 2001, Distelhorst and Hughes 2003, McKinnon et al. 2008, Tabet et al. 2008

2.3 Timolol

2.3.1 Systemic use of timolol

Timolol is a non-selective moderately lipophilic β-adrenergic antagonist which has been used in oral formulation for the treatment of hypertension and prophylaxis of migraine (Dunn and Frohlich 1981, Dollery et al. 1991, Blumenfeld 2005). Other indications are angina pectoris and secondary prevention of myocardial infarction (Dollery et al. 1991). Treatment is usually started with 10 mg once daily or 5 mg twice daily and the dose may be increased if required. The bioavailability of timolol after oral dosing is about 75% (Meier 1982); distribution volumes of 1.74 –3.64 l/kg have been reported (Dollery et al. 1991). The plasma protein binding varies between 10 and 80% (Dollery et al. 1991). A half life from 2 to 5 hours has been reported. Timolol is metabolized extensively in the liver, but 20% is excreted unchanged by the kidney. The use of oral preparations has been diminished; today e.g. in Finland there are no marketing authorizations for oral timolol (National Agency for Medicines Finland, web search for products).

2.3.2 Ophthalmic use of timolol

Topically timolol has been used for the treatment of glaucoma and increased IOP since 1977, when it was shown to be an effective ocular hypotensive agent (Zimmerman et al. 1977, Brooks and Gillies 1992). It is still recommended by the European Glaucoma Society as the first choice in treating glaucoma together with prostaglandin analogs and α-adrenergic agonists (European Glaucoma Society 2008).

Timolol lowers the IOP by reducing the formation of aqueous humor (Neufeld et al. 1983). In the normal human eye, timolol has been reported to bring about a 13 to 48% decrease in the production of aqueous humor, the mean suppression being 34% (Coakes and Brubaker 1978). In chronic timolol users IOP has been reported to be 15% lower and aqueous humor production 24% lower during maintenance treatment than one month after treatment discontinuation (Schlecht and Brubaker 1988). After long-term treatment (mean duration 44 months) the effect of timolol on IOP persists for 2 to 4

weeks following its withdrawal. Topical timolol formulations are used in order to minimize systemic effects and toxicity as well as to maximize the local concentration (Edeki et al. 1995).

2.3.3 Local adverse effects of timolol

In most patients ophthalmic timolol eye drops are topically well tolerated (Brooks and Gillies 1992). Most commonly reported adverse ocular effects are irritation and blurred vision (Table 3) (Zimmerman et al. 1983). Local hypersensitivity reactions may occur as with all topical medications (Brooks and Gillies 1992). Hyperemia, allergic blepharoconjunctivitis, keratitis, superficial punctate keratitis and corneal anesthesia have been reported (van Buskirk 1980, Zimmerman et al. 1983). Dry eyes may be associated with ocular administration of timolol.

Some of the local adverse effects may be related to the formulation of the ophthalmic product rather than the active substance timolol. The most commonly used preservative in eye drop products is benzalkonium chloride (Jaenen et al. 2007). Patients with preserved glaucoma medications more often experience pain or discomfort, foreign body sensation, stinging or burning and dry eye sensation as compared with those using preservative-free products. It has also been reported that repeated or long-term instillation of preservatives may lead to hypersensitivity reactions (Baudoiun 2005).

2.3.4 Systemic adverse effects of timolol

Topically applied timolol is absorbed not only into the eye, but also systemically. It has been reported that an approximately 80% portion of a topically administered eye drop drains through the nasolacrimal duct and is systemically absorbed (Shell 1982). Recently similar findings have been reported with aqueous timolol 0.5% (Korte et al. 2002). According to early clinical trials timolol was reported to be an effective agent without the adverse effects associated with other antiglaucoma agents on the market (Nelson et al. 1986). However, persons with underlying cardiovascular and respiratory diseases were excluded in most clinical trials. Shortly after timolol became available on

the market, adverse reactions such as severe cardiac and respiratory events were reported associated with ophthalmic timolol (Table 3).

Cardiovascular events caused by timolol are adrenergic β-antagonist effects (van Buskirk 1980, Zimmerman et al. 1983, Nelson et al. 1986). These effects include bradycardia, arrhythmia, hypotension and syncope. Due to these potential severe adverse effects it should be ascertained that a patient has no contraindications such as severe bradycardia before prescribing timolol. Even though the cardiovascular effects and contraindications of timolol are known, reports of serious adverse events are still published. Recently Müller and collegues (2006) advised doctors to be cautious when prescribing timolol for patients with pre-existing cardiovascular diseases. They described three cases of patients 73 to 74 years who due to timolol eye drops had experienced serious episodes of syncope and falls leading to injury such as bone fractures. These types if injuries may involve a very substantial economic burden (Järvinen et al. 2008).

Timolol causes pulmonary effects such as bronchospasm, respiratory failure and increased cough (van Buskirk 1980, Zimmerman et al. 1983, Nelson et al. 1986, Brooks and Gillies 1992). Bronchoconstriction resuls from antagonism of β_2-receptors caused by timolol. Like cardiovascular diseases also pulmonary diseases should be taken into account before starting a new patient on timolol treatment. Timolol is contraindicated in patients with a history of asthma or other chronic pulmonary diseases.

Timolol use is also associated with adverse effects in other organs. Central nervous system reactions, e.g. confusion, dizziness, fatigue and headache as well as gastrointestinal and dermatologic effects have been reported (van Buskirk 1980, Zimmerman et al. 1983).

Table 3. Adverse effects reported for ophthalmic timolol.

LOCAL ADVERSE EFFECTS	SYSTEMIC ADVERSE EFFECTS
Ocular	**Cardiovascular**
allergic blepharoconjunctivitis	angina
corneal anesthesia	arrhythmia
diplopia	bradycardia
dry eyes	cerebrovascular accident
hyperemia	heart failure
keratitis	hypotension
photophobia	palpitations
superficial punctate keratitis	syncope
visual field defect	**Central nervous system**
	anxiety
	asthenia
	confusion
	decreased libido
	depression
	dizziness
	fatigue
	hallucinations
	headache
	malaise
	phychosis
	sensory disorders
	somnolence
	Dermatologic
	alopecia
	rash
	urticaria
	Gastrointestinal
	cramps
	diarrhea
	dyspepsia
	nausea
	vomiting
	Pulmonary
	apnea
	bronchospasm
	cough
	dyspnea
	respiratory failure

References: van Buskirk 1980, Zimmerman et al. 1983, Nelson et al. 1986, Brooks and Gillies 1992.

2.3.5 Ophthalmic timolol formulations

Timolol eye drops are available mainly as aqueous eye drops in concentrations of 0.25% and 0.5% (Brooks and Gillies 1992). Both concentrations of conventional aqueous eye drops are administered twice daily. As noted above, a large portion of the topically administered eye drop is absorbed systemically. Topical ophthalmic administration can be compared to an intravenous bolus: first-pass hepatic metabolism is avoided and plasma kinetics and systemic effects are similar (Korte et al. 2002, Lama 2002). In order to minimize adverse cardio-pulmonary effects novel timolol formulations have been developed (Dickstein and Aarsland 1996, Mayer and von der Ohe 1996, Niño et al. 2002, Uusitalo et al. 2005). Gel formulations with 0.5% timolol and reduced 0.1% timolol concentrations have been introduced. Timolol 0.1% hydrogel administered once daily has been shown to be clinically as effective as conventional aqueous 0.5% timolol administered twice daily (Rouland et al. 2002). On the other hand, the risk of adverse cardiac effects has been shown to be reduced (Uusitalo et al. 2005, Uusitalo et al. 2006).

2.4 Pharmacokinetics of topical ophthalmic drugs

The topical ophthalmic route is a noninvasive, convenient and effective mode of drug delivery to the anterior segment of the eye (Amrite et al. 2008, Ghate and Edelhauser 2008). This route is useful for the treatment of diseases of the anterior segment or for delivery of drugs whose site of action lies in this section. The target of activity can be extraocular, e.g. when treating conjunctivitis, or intraocular when treating e.g. glaucoma; in this case corneal passage of the drug is required (Shell 1982). It has been reported that generally the ocular bioavailability of ophthalmic drugs is less than 10% (Attar et al. 2005). There are suggestions that only 1-7% of the topically administered dose reaches the aqueous humor; after administration there are several steps which diminish the bioavailability of the drug (see Figure 5) (Ghate and Edelhauser 2008).

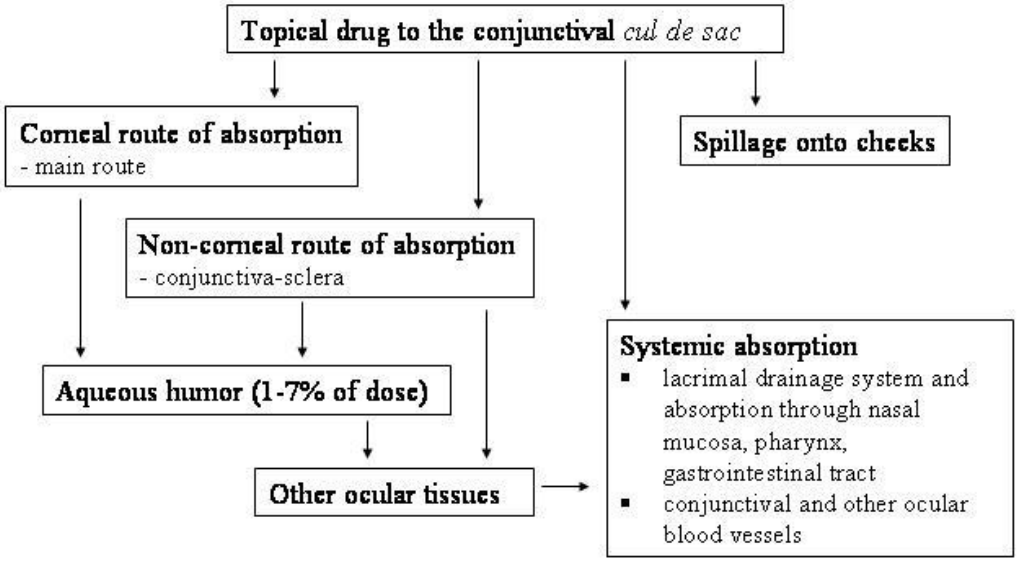

Figure 5. Typical routes of a topically applied ophthalmic drug (modified from Ghate and Edelhauser 2008).

The volume of a commercial eye drop is between 25 and 75 µl (Shell 1982, Ghate and Edelhauser 2008). The normal tear volume in the conjunctival *cul de sac* is 7 - 9 µl. The estimated maximum volume which the *cul de sac* can contain is 30 µl. After administration of an eyedrop the momentary increase in volume and the irritative properties of the drug cause a blinking reflex and increased tear secretion. Most of the administered drug is quickly eliminated from the *cul de sac* though the lacrimal drainage system and the excess is spilled onto the cheeks.

The main route of a drug from the *cul de sac* to the anterior segment of the eye is the corneal (Ghate and Edelhauser 2008). This is the route especially for small and lipophilic drugs. The corneal epithelium is the component of the corneal barrier most resistant to drug penetration. Lipophilic drugs penetrate the corneal epithelium through the cells by intracellular route and hydrophilic drugs or ions of small molecular weight through the paracellular route passing between the cells. For large hydrophilic molecules the conjunctival-scleral layer has a role in absorption.

The systemic absorption via the conjunctival, nasal, oropharyngeal and gastrointestinal mucosa may result in plasma levels sufficient to cause systemic adverse effects and interactions with other drugs (Gerber et al. 1990). As much as 80% or more of an administered eyedrop is absorbed systemically, and this systemically absorbed dose avoids the first pass metabolism (Shell 1982). Topical administration thus resembles more intravenous than oral administration and topically administered ophthalmic drugs can attain relatively high plasma levels (Gerber et al. 1990).

A drug administered into the body via different routes (e.g. orally, inhalation, nasal, or the ophthalmic route) is absorbed, distributed and metabolized before being eliminated from the body. To be finally eliminated a drug must in most cases be transformed into a more polar water-soluble and excretable metabolite (Meyer 1996). The major organ for this biotransformation is the liver. Also other organs, expecially the intestine and kidneys, lung and skin, can contribute to drug metabolism. Two metabolic routes, phase I and phase II reactions, are responsible for the transformation of the majority of drugs in use (Wijnen et al. 2007). Phase I reactions are executed mainly by cytochrome P450 enzymes and involve hydroxylation, reduction and oxidation of substances. Phase II reactions involve glucuronidation, sulfation, acetylation or methylation of substrates.

To summarize

Timolol is a drug widely used for lowering increased IOP. It is an effective treatment mode, but as much as 80% of the ophthalmic dose can be absorbed into the systemic circulation after administration of traditional aqueous timolol eye drops. This may lead to plasma levels high enough to cause adverse systemic effects such as bradycardia, lowering of blood pressure and bronchoconstriction. Recently, a gel formulation of timolol has been developed in order to improve ophthalmic absorption and systemic safety. The metabolism of timolol, which will be reviewed in the following, may also affect the safety of timolol in the treatment of glaucoma.

2.5 Cytochrome P450 (CYP) enzymes

The cytochrome P450 (CYP) enzymes are a superfamily of heme-containing enzymes (Conney 1982, Guengerich 1992, Sikka et al. 2005). These enzymes are monooxygenases or mixed function oxidases which metabolize endogenous substances such as corticosteroids and fatty acids as well as foreign compounds such as food components, carcinogens and drugs. Each P450 enzyme is encoded by a different gene. Enzymes are divided into families, subfamilies and isoforms based on amino acid sequence identity (Figure 6) (Attar et al. 2005, Sikka et al. 2005, Wijnen et al. 2007). CYP enzymes with 40% homology in amino acid sequences form a CYP family identified with an arabic number. A subfamily is a group of enzymes having at least 55% amino acid sequence homology. The subfamily is designated with a capital letter. This subfamily letter is followed by an arabic number designating the specific member, the individual enzyme, in that subfamily. An example of the cytochrome P450 enzyme nomenclature is given in Figure 7.

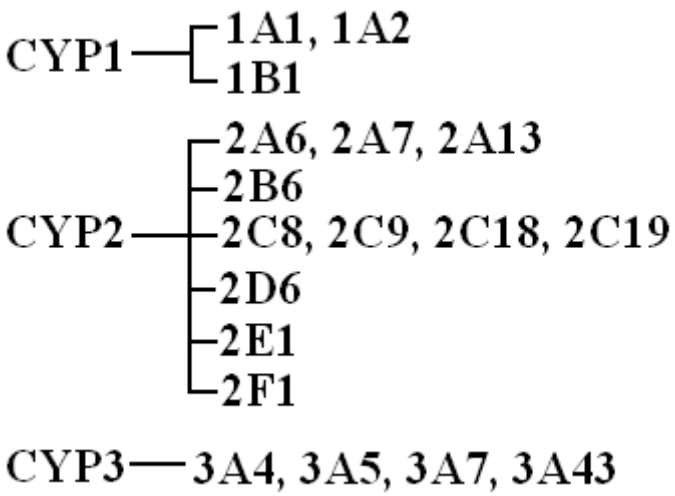

Figure 6. Cytochrome P450 enzymes in families 1 – 3 (modified from Ingelman-Sundberg and Rodriguez-Antona 2005).

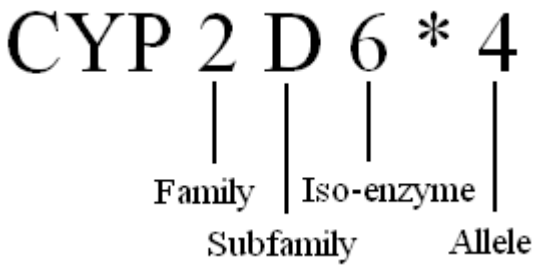

Figure 7. An example of the nomenclature of the cytochrome P450 enzymes (Wijnen et al. 2007).

Altogether 115 cytochrome P450 genes have been identified in the human genome, 57 active genes and 58 pseudogens (Ingelman-Sundberg and Rodriguez-Antona 2005, Wijnen et al. 2007). These enzymes form 18 families (Nebert and Russel 2002). The cytochrome P450 enzymes in families 1-3 are responsible for 70-80% of all phase I-dependent metabolism of drugs in clinical use and also participate in the metabolism of many other foreign compounds (Figure 8) (McKay 1993, Ingelman-Sundberg and Rodriguez-Antona 2005, Wijnen et al. 2007). These enzymes are located mainly along the membrane of the endoplasmic reticulum of the cells of almost every major organ, being however most highly expressed in the liver (Attar et al. 2005, Sikka et al. 2005). CYP enzymes are also active in several other tissues such as the small intestine, lung and kidney. The majority of the reactions catalyzed occur in the liver. A drug can either be a substrate for only one CYP enzyme or it can be metabolized by several CYP enzymes.

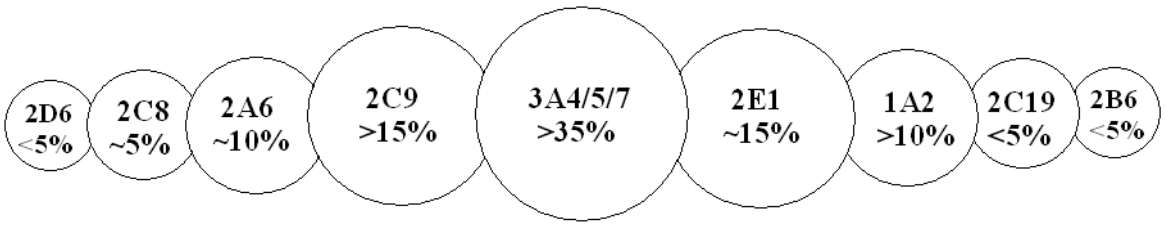

Figure 8. Relative abundance of individual CYP forms in the liver (modified from Pelkonen et al. 2008). Note: Relative abundancies in the liver are not the same as percentage of drugs which are substrates for each CYP enzyme.

2.5.1 Polymorphism of CYP enzymes

CYP enzymes are polymorphic enzymes, meaning that mutations in the *CYP* genes encoding CYP enzymes may produce enzyme products with changed enzyme activity (Meyer and Zanger 1997, Pelkonen et al. 1998, Ingelman-Sundberg and Rodriguez-Antona 2005, Sikka et al. 2005). An enzyme is considered genetically polymorphic when variant genes are present in at least 1% of a population. The activity of the enzyme products can be abolished, reduced, altered or increased, leading to four major phenotypes:

- Poor metabolizers (PMs) are homozygous for the recessive allele, the phenotype, in consequence of the lack of a functional enzyme.
- Intermediate metabolizers (IMs) are heterozygous for a defective allele.
- Extensive metabolizers (EMs) are homozygous or heterozygous for the dominant allele; they carry two functional gene copies.
- Ultrarapid metabolizers (UMs) have more than two functional gene copies.

The frequency of phenotype distribution varies in different ethnic groups. In the CYP2C family, e.g. the frequency of the poor metabolizer phenotype is 3-5% in Caucasians, but 18-23% in Oriental populations (Goldstein and de Morais 1994). It has been surmized that 15-20% of all drug treament is influenced by the polymorphism of *CYP* genes (Meyer and Zanger 1997, Pelkonen et al. 1998, Ingelman-Sundberg and Rodriguez-Antona 2005, Sikka et al. 2005). The most important polymorphic CYP enzymes are

CYP2C9, CYP2C19 and CYP2D6, as all have a large variety of drugs as substrates. Plasma levels after a drug dose may differ severalfold between patients with different enzyme phenotype. For example the dosage of the tricyclic antidepressant nortriptyline should be 30 -50 mg in CYP2D6 PMs and as high as 500 mg in CYP2D6 UMs in order to achieve the same plasma levels. Adverse drug reactions are also often associated with the patient's CYP genotype.

2.5.2 CYP induction

Adverse drug reactions may also be mediated by CYP enzyme induction (Sikka et al. 2005). CYP enzyme activity may be increased through induction, this being a consequence of an increased amount of enzyme molecules (Pelkonen et al. 1998, Sikka et al. 2005). Several human CYP enzymes are known to be inducible by drugs, often other than the substrates of the specific enzyme in question (Guengerich 1992). In addition to drugs some dietary components and smoking can also result in enzyme induction. This process is mediated to a major extent by ligand-activited transcription factors involving aryl hydrocardon receptor (AHR), AHR nuclear translocator (ARNT), pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor (GR) and retinoid X receptor (RXR) (Pelkonen et al. 2008). Induction has not been reported for all CYP enzymes; of the major enzymes CYP1A2, CYP2C9, CYP2C19, CYP2E1 and CYP3A4, but not CYP2D6, are known to be inducible (Sikka et al. 2005, Pelkonen et al. 2008). The increased amount of enzyme molecules in induction may be a result of increased transciption and translation or of the stabilization of an enzyme, which leads to a reduction in the natural rate of enzyme breakdown (Pelkonen et al. 1998, Sikka et al. 2005). As the process leading to this increased amount of enzyme activity takes time, there is a certain lag phase before induction can be observed. Rifampicin, an inducer of several drug-metabolizing enzymes, has the greatest effect on the expression of CYP3A4 (Niemi et al. 2003). Full induction of enzymes has been reported to be reached in about one week after treatment commences. After discontinuation of rifampicin the induction dissipates in approximately two weeks. The antiepilectic drugs carpamazepine and phenytoin are also clinically

significant inducers of several CYP enzymes, including CYP1A2, CYP2C9, CYP2C19 and CYP3A4 (Perucca 2006).

2.5.3 CYP inhibition

CYP enzyme activity may be reduced through inhibition, in which the function of the enzyme is prevented (Sikka et al. 2005). CYP enzyme inhibition can be either reversible or irreversible (Table 4), reversible inhibition being the more common type. It is transient and enzyme activity reverts to its normal level once the inhibitor has been cleared. Reversible inhibition can be divided into competitive, non-competitive, uncompetitive and mixed-type inhibition (Pelkonen et al. 2008). In competitive inhibition substrate and inhibitor compete to bind to the same position on the active site of the enzyme. In non-competitive inhibition the active binding sites of these two are different. Uncompetitive inhibition consists in the inhibitor binding to the enzyme-substrate complex, not to the free enzyme entity. Mixed-type of inhibition has elements of both competitive and noncompetitive inhibition.

The intrinsic clearance (CL_{int}) is the ratio of the maximal velocity of metabolite formation (V_{max}) and Michaelis-Menten constant (K_m), $CL_{int} = V_{max}/K_m$ (Pelkonen et al. 1998, Guengerich 1999). The form of reversible inhibition has different effects on the values of V_{max} and K_m. In competitive inhibition V_{max} remains unchanged and K_m increases. In noncompetitive inhibition V_{max} decreases but K_m is unchanged. In uncompetitive inhibition V_{max} and K_m are decreased proportionally and the ratio V_{max}/K_m remains constant.

Irreversible inhibition, also known as mechanism-based inhibition, may occur through the formation of metabolite intermediate complexes or through strong covalent binding of reactive intermediates to the protein or heme of the CYP (Sikka et al. 2005, Pelkonen et al. 2008). Irreversible inhibition is long-lasting and only resynthesis of the enzyme can restore enzyme activity.

Table 4. The forms of cytochrome P450 inhibition.

Form of inhibition	Typical for inhibition
Reversible inhibition competitive non-competitive uncompetitive mixed-type	Transient Enzyme activity is restored after the inhibitor has been eliminated.
Irreversible inhibition	Long-lasting Enzyme activity is restored only after resynthesis of the enzyme.

References: Sikka et al. 2005, Pelkonen et al. 2008.

2.5.4 CYP1 family

The CYP1 gene family has three members in two subfamilies, CYP1A and CYP1B (Nebert and Russel 2002). CYP1 gene family expression can be induced via the aryl hydrocarbon receptor (AHR) by polycyclic aromatic hydrocarbons found e.g. in cigarette smoke or grilled food. CYP1A1 and CYP1B1 are expressed mainly in extrahepatic tissues (Pelkonen et al. 1998). The expression of CYP1A1 has been detected e.g. in the human placenta and breast (Hakkola et al. 1996, Masson et al. 2005). CYP1B1 expression has been found e.g. in the ovary, adrenal gland, breast, uterus and prostate (Chun et al. 2001). CYP1A2, on the other hand, is found mainly in the liver, but can also be expressed in some extrahepatic tissues such as the small intestine (Granfors et al. 2004). CYP1A2 has been estimated to be responsible for 5% of drug metabolism in phase I reactions (Nebert and Russel 2002, Ingelman-Sundberg and Rodriguez-Antona 2005). Substrates for CYP1A2 metabolism are e.g. caffeine, tizanidine and theophylline (Pelkonen et al. 1998, Backman et al. 2006) The other enzymes in this gene family, CYP1A1 and CYP1B1, seem not to have a significant role in drug metabolism (Nebert and Russel 2002). All three enzymes are known to detoxify or activate many environmental carcinogens. CYP1B1 is important not only as a metabolizing enzyme but also in the development of glaucoma; mutations in the *CYP1B1* gene have been reported to cause congenital glaucoma (Vasilou and Gonzalez 2008).

2.5.5 CYP2 family

The CYP2 family is the largest P450 family in mammals, consisting of 13 subfamilies and altogether 16 different genes (Nebert and Russel 2002). The human genome contains several genes which belong to the CYP2C subfamily (Pelkonen et al. 1998). CYP2C enzymes CYP2C8, CYP2C9, CYP2C18 and CYP2C19 metabolize over half of all drugs (Nebert and Russel 2002).

CYP2C9 is present in several tissues, e.g. in the kidney, testes, prostate, ovary and duodenum (Wijnen et al. 2007). However, most of the CYP2C9 activity in drug metabolism takes place in the liver. CYP2C9 is a polymorphic enzyme, a circumstance which should be taken into account when using substrates of this enzyme, since many of the substrates have a narrow therapeutic window (Sikka et al. 2005). Substrates include warfarin, tolbutamide and phenytoin as well as many nonsteroidal anti-inflammatory agents and angiotensin II receptor blockers. CYP2C9 inhibitors are e.g. fluconazole and metronidazole.

CYP2C19, which is found mainly in the liver, accounts with CYP2C9 for approximately 20% of the total cytochrome P450 activity (Wijnen et al. 2007). Polymorphism of this enzyme differs between ethnic groups. The PM phenotype occurs in 2-6% of Causasians and in as many as 15-30% of Asians. The prevalence of EMs in Caucasians has been reported to be over 70% and that of IMs 20%. Known CYP2C19 substrates are diazepam, citalopram, amitriptyline, moclobemide, phenytoin and proton pump inhibitors omeprazole, esomeprazole, lanzoprazole and pantoprazole (Lee et al. 2005, Wijnen et al. 2007, Pelkonen et al. 2008). The CYP2C19 substrate omeprazole has also been reported to be its inhibitor. Fluvoxamine, ticlopidine and fluoxetine are less selective inhibitors of CYP2C19. Oral contraceptives are moderate inhibitors of CYP2C19.

CYP2D6 is expressed mainly in the liver, but expression has also been reported in other tissues, including the lung, heart, brain and prostate (Frank et al. 2007, Wijnen 2007). CYP2D6 has been reported to metabolize about 20-30% of all available drugs (Mulder et al. 2007). The enzyme activity has a wide range of interindividual and interethnic

variability, caused mainly by genetic polymorphism. The frequency of PMs has been reported to be 3-10% in Caucasians, less than 2% in Chinese, Japanese, African and American ethnic groups and 3.2% in Finns (Arvela et al. 1988, Fishbain et al. 2004). CYP2D6 gene duplication causing UMs has been reported in 20% of Ethiopians, 7% of Hispanics, 1-10% of Caucasians, and only 1.5% of Scandinavians (Fishbain et al. 2004, Mulder et al. 2007). CYP2D6 polymorphism can cause from 30- to 40-fold differences in substrate clearance, resulting substantial differences in plasma levels and plasma levels outside the therapeutic range (Frank et al. 2007). Polymorphism should therefore be taken into account when dosing drugs metabolized by CYP2D6. Substrates of CYP2D6 enzyme are e.g. metoprolol, spartaine, dextromethorphan and timolol (Edeki et al. 1995, Ishii et al. 2000, Nieminen et al. 2007, Wijnen et al. 2007, Goryachkina et al. 2008, Pelkonen et al. 2008). Inhibitors include paroxetine, fluoxetine, sertaline, cimetidine, quinidine and terbinafine.

CYP2E1, though one of the most abundant hepatic CYP enzymes, has no significant role in the metabolism of drugs (Pelkonen et al. 2008). Only a few drugs are infact metabolized by this enzyme, one of most important being paracetamol (acetaminophen) (Sikka et al. 2005). CYP2E1 has a central role in the metabolism of ethanol, and it has also been associated to paracetamol-related hepatotoxicity (Pelkonen et al. 2008). Other substances metabolized by this enzyme are certain carcinogens and other xenobiotics (Pelkonen et al. 1998).

2.5.6 CYP3 family

The CYP3 family has four members, CYP3A4, CYP3A5, CYP3A7 and CYP3A43 in one subfamily (Nebert and Russel 2002). CYP3A is the most highly expressed P450 family in the liver, being reported to comprise up to 60% of total hepatic P450 content (Pelkonen et al. 1998, McCune et al. 2000, Sikka et al. 2005). It is also a family mostly present in the small intestine. CYP3A isoforms have been estimated to contribute to the metabolism of more than 50% of all therapeutic agents currently in use. The function of hepatic CYP3A43 is not yet known (Nebert and Russel 2002).

The interindividual variablity in hepatic and intestinal CYP3A activity has been reported to be considerable, with differences 5- to 10-fold (McCune et al. 2000). Also the expression of CYP3A family members in the human liver has been reported to differ between individuals: CYP3A4 is expressed in all individuals, CYP3A5 protein is expressed in detectable levels in about 25% of individuals and CYP3A7 is expressed particularly in the fetal liver, but not in adults (Pelkonen et al. 1998, Wilkinson 2005). The induction of expression of CYP3A genes is mediated by pregnane X receptor (PXR), a transcription activator factor present in the cytosolic fraction of cells (Pascussi et al. 2000, Tang et al. 2005, Trubetskoy et al. 2005). PXR can be activated by a number of different steroids such as glucocorticoids, and drugs including rifampicin, clotrimazole, nifedipine and phenobarbital.

While the substrate and inhibitor specificity of CYP3A5 would appear to be quite similar to CYP3A4, the catalytic capability might be lower (Pelkonen et al. 2008). CYP3A4 has a pivotal role in drug metabolism. Substrates are e.g. cyclosporine A, erythromycin, nifedipine, midazolam and triazolam. Also lipid-lowering drugs simvastatin, lovastatin and atorvastatin are metabolized by CYP3A4 (Neuvonen et al. 2006). Dexamethasone and rifampin are inducers of this enzyme and ritonavir, itraconazole and ketoconazole are CYP3A4 inhibitors (Backman et al. 1996, McCune et al. 2000, Neuvonen et al. 2006). In addition to drugs, the diet may have an important role in CYP3A4 interactions, as furanocoumarins and flavones of grapefruit juice are clinically relevant inhibitors of this enzyme (Schmiedlin-Ren et al. 1997, Lilja et al. 2004, Pelkonen et al. 2008).

To summarize

Cytochrome P450 enzymes have an important role in the metabolism of most drugs used today. Some of these enzymes are polymorphically expressed, i.e. individuals may have different phenotypes causing different enzyme activities. Poor, intermediate, extensive and ultrarapid metabolizers handle substrates of the specific CYP enzyme with different activities, which means that the same drug dose may lead to high inter-individual variations in plasma concentrations. Other important factors in the metabolism are

induction and inhibition of CYP enzymes by other drugs, which may cause wide variations in the metabolism of drugs, this possibly leading to adverse effects.

2.5.7 CYP enzymes in the eye

There is only limited information of the expression of CYP enzymes in the human eye. Expression of CYP1A1, CYP2C, CYP2E1 and CYP3A5 in human corneal epithelial biopsies and in immortalized human corneal epithelial cells has been reported (Table 5) (Offord et al. 1999). Of the CYP2C enzymes CYP2C18 is most abundantly expressed, whereas CYP2C8 and CYP2C9 are expressed at a lower level. Expression of CYP1B1 has been detected at mRNA and/or protein level in several human ocular tissues (Attar et al. 2005, Doshi et al. 2006). Expression of CYP2A6, CYP2C8, CYP2D6, CYP2E1 and CYP3A4 at very low levels has also been reported (Zhang et al. 2008).

In some other species, expression of the CYP enzymes has also been reported (Table 5). CYP1A1 and CYP1A2 have been detected in the mouse ciliary and iris epithelium (Attar et al. 2005). CYP1A and also CYP3A expression has also been found in the rabbit ocular tissues. In the rat lens expression of CYP2B1/2 and CYP2C11 has been observed.

Table 5. CYP enzymes of families 1 to 3 expressed at mRNA or protein level even at very low levels in ocular tissues.

CYP enzyme	Ocular tissue	Species	Ref.
CYP1A	Conjunctiva	Rabbit	4
	Corneal epithelium	Human	3
	Ciliary epithelium	Mouse	4
	Iris-ciliary body	Rabbit	4
	Iris epithelium	Mouse	4
	Lacrimal gland	Rabbit	4
CYP1B	Ciliary body	Human	1, 2
	Cornea	Human	2
	Iris	Human	1,2
	Non-pigmented ciliary epithelial cell line	Human	2
	Retina	Human	1, 2
	Retinal pigment epithelium	Human	2
	Trabecular meshwork	Human	1, 2
CYP2A	Choroid-retina	Human	5
	Cornea	Human	5
	Iris-ciliary body	Human	5
CYP2B	Lens	Rat	4
CYP2C	Choroid-retina	Human	5
	Ciliary body	Mouse	4
	Cornea	Human, mouse	5, 4
	Corneal epithelium	Human	3
	Lens	Mouse, rat	4
	Retina	Mouse	4
CYP2E	Choroid-retina	Human	5
	Cornea	Human	5
	Corneal epithelium	Human	3
	Iris-ciliary body	Human	5
CYP2D	Choroid-retina	Human, rabbit	5
	Conjunctiva	Rabbit	4
	Cornea	Human	5
	Iris-ciliary body	Human, rabbit	5, 4
	Lacrimal gland	Rabbit	4
CYP3A	Conjunctiva	Rabbit	4
	Cornea	Human	5
	Corneal epithelium	Human	3
	Iris-ciliary body	Human, rabbit	5, 4
	Lacrimal gland	Rabbit	4

References: [1]Sarfarazi 1997, [2]Stoilov et al. 1998, [3]Offord et al. 1999, [4]Attar et al. 2005, [5]Zhang et al. 2008.

2.6 Studying drug metabolism and drug-drug interactions

Drug development is a long and time-demanding process during which the characteristics of a drug candidate need to be identified in order to develop a preparate both effective and safe in use. Before a drug candidate can be administered to a human subject for the first time a great deal of information on its properties, e.g. its metabolism, is required. *In vitro* systems are widely used in research for measuring drug metabolism and interactions (Pelkonen and Raunio 2005). Metabolic stability (disappearence of a chemical in human liver preparations), identification of metabolites and enzymes catalyzing metabolic routes, enzyme kinetic characterization of principal metabolic reactions and the affinity of a chemical for CYP or other enzymes are among the aspects generally investigated for a new chemical entity. Nowadays, regulatory guidelines require specific *in vitro* and/or *in vivo* drug-drug interaction studies to be conducted for any new drug molecule during its development (Bjornsson et al. 2003, Pelkonen and Raunio 2005).

Human liver preparations such as recombinant expressed P450s, microsomes, hepatocytes and liver slices are widely used models for *in vitro* metabolism studies (Table 6) (Andersson et al. 2001, Takashima et al. 2005, McGinnity et al. 2006). Microsomes are used expecially in CYP-inhibition studies and together with liver homogenates they are the most widely employed *in vitro* systems in drug metabolism studies (Pelkonen and Turpeinen 2007). Microsomes are derived from the endoplasmic reticulum and contain both CYPs and UDP-glucuronyl transferases (UGTs). They are easy to repair and readily available. A disadvantage is however loss of some enzyme activity during preparation.

The hepatocytes bring research one step closer to the human liver. They contain the full complement of xenobiotic metabolizing enzymes and transporters which a drug may encounter during first pass metabolism (McGinnity et al. 2006, Soars et al. 2007). The absence of a supply of good-quality human tissue, however, limits their use in routine screening (McGinnity et al. 2004). One solution to this problem is afforded by cryopreserved hepatocytes, which are continuously available. Commercially available

cryopreserved hepatocytes from several donors have a similar range of oxidation and glucuronidation rates compared with freshly isolated hepatocytes. As compared with these, cryopreserved hepatocytes possess enzyme activity for a shorter period of time (Pelkonen and Turpeinen 2007). However, the pattern of metabolite formation is similar using either system (McGinnity et al. 2004).

In vivo predictions based on *in vitro* results save drug development costs and time while also reducing the number of animals needed for absorption, distribution, metabolism and excretion studies (Salonen et al. 2003). Prediction of drug-drug interactions based on *in vitro* experiments is fairly reliable (Pelkonen and Raunio 2005). However, it should be taken into consideration that pharmacokinetic interaction *in vitro*, even though well documented, is not necessarily clinically significant (Bjornsson et al. 2003), and data from clinical trials and postmarketing surveillance give more reliable information as to the clinical relevance of pharmacokinetics and drug interactions in humans.

Table 6. Comparison of human enzyme sources used in *in vitro* studies.

Enzyme source	**Enzyme content**	**Availability**	**Disadvantages**
Liver homogenate	All phase I and II hepatic enzymes	Relatively good, commercially available	Liver architecture lost, cofactor addition necessary
Microsomes	Phase I enzymes (CYPs) and UDP-glycuronosyl transferases	Relatively good, commercially available	Cofactor addition necessary
cDNA-expressed individual CYP enzymes	Individual CYPs	Commercially available	Only one enzyme can be studied at a time
Primary hepatocytes	Whole complement of drug-metabolizing enzymes cellularly integrated	Difficult to obtain, commercially available	Requires specific techniques and well-established procedures
Cryopreserved hepatocytes	Whole pattern of drug-metabolizing enzymes present	Continuously available, commercially available	The period of enzyme activity limited
Immortalized cell lines	Unlimited source of enzymes	Available on request	Expression of most drug-metabolizing enzymes is poor
Liver slices	Whole complement of drug-metabolizing enzymes and cell-cell connections	Difficult to obtain, fresh tissue needed	Requires specific techniques and well-established procedures

References: modified from McGinnity et al. 2004, Pelkonen and Turpeinen 2007.

3 AIMS OF THE STUDY

This study set out to investigate the metabolism of the IOP-lowering drug timolol and the drug-drug interactions of timolol with commercially available medicines. Glaucoma patients are often elderly and may be using several other drugs concomitantly with an ophthalmic timolol product. The expression of drug-metabolizing CYP enzymes in the ocular tissue was investigated and the metabolism and interaction potential of timolol characterized. The effects of two different timolol formulations, eye drops and eye gel, were investigated in terms of timolol pharmacokinetics and its potential for interaction with CYP2D6 inhibitors. Thereafter, the metabolism of timolol in the human eye was investigated.

The aims of the individual studies were:

- to investigate the expression of P450 enzymes in human nonpigmented ciliary epithelial cells *in vitro* (Study I).

- to identify timolol metabolites and to determine the P450-associated metabolic and interaction possibilities of timolol (Study II).

- to investigate the effects of CYP2D6 and CYP2C19 inhibitors on timolol metabolism *in vitro* (Study III).

- to compare the penetration of timolol into the human aqueous humor after administration of timolol 0.1% eye gel and aqueous 0.5% timolol eye drops (Study IV).

4 MATERIALS AND METHODS

Detailed descriptions of the materials and methods are to be found in the original publications.

4.1 Expression of cytochrome P450 enzymes in human ciliary epithelial cells

4.1.1 Chemicals

Dexamethasone, Dulbecco modified Eagle medium and TRI reagent® RNA Isolation Reagent were purchased from Sigma-Aldrich (Steinheim, Germany). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from the National Cancer Institute Chemical Carcinogen Repository (Bethesda, MD, USA). The First Strand cDNA synthesis kit was from Amersham Biosciences (Buckinghamshire, UK). Ethidium bromide, agarose NA® and agarose Prep® were purchased from Amersham Biosciences AB (Uppsala, Sweden) and the Qiaquick Gel Extraction Kit from Qiagen GmbH (Hilden, Germany).

PCR reaction buffer, dNTP reaction mixture and DyNAzyme DNA polymerase were obtained from Finnzymes (Espoo, Finland). Sense and antisense primers were from Sigma-Genosys (Cambridge, UK) and CYP1B1 Supersomes from BD Biosciences (San Jose, CA, USA). Secondary peroxidase-conjugated goat antirabbit antibody and chemiluminescent peroxidase substrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tfx-20 reagent® for transfection of eukaryotic cells, the control reporter plasmid: thymidine kinase promoter-Renilla luciferase reporter plasmid (pRL-TK) and Dual-Luciferase Reporter Assay System were from Promega (Madison, WI, USA). Opti-MEM I medium and fetal bovine serum were obtained from Invitrogen (Paisley, Scotland, UK).

4.1.2 Human ciliary epithelial cells and their induction

The nonpigmented human ciliary cell line was a generous gift from Professor Miguel Coca-Prados, Yale University, New Haven, CT, USA. The cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in standard conditions at 37 ˚C, 5% CO_2 and saturated humidity (95%). To study induction nearly confluent cells were treated with dexamethasone and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 24 h. After exposure the cells were collected and used for RNA or protein extraction. Total RNA was extracted with TRI-reagent and complementary DNA (cDNA) was synthesized with a cDNA synthesis kit (First Strand).

4.1.3 Expression of mRNAs

The expression of mRNAs of drug metabolizing CYP enzymes from family 1: CYP1A1, CYP1A2 and CYP1B1, family 2: CYP2A6, CYP2B6, CYP2C8-19, CYP2D6 and CYP2E1 and family 3: CYP3A4, CYP3A5 and CYP3A7 as well as the nuclear receptors constitutive androstane receptor (CAR), pregnane X receptor (PXR) and glucocorticoid receptor (GR), and the basic helix-loop-helix factors aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) was studied by a reverse transcriptase-polymerase chain reaction (RT-PCR) method. Each PCR reaction mixture contained cDNA, PCR reaction buffer, dNTP reaction mixture, sense and antisense primer, DNA polymerase and water. After PCR amplification, the reaction mixture was electrophoresed into 0.5% agarose gel and stained with ethidium bromide.

For identification of CYP2D6 PCR products a low-melting-point agarose gel using Agarose Prep was prepared and the CYP2D6 RT-PCR products run on the gel. The three most promiment bands were extracted from the gel using the Qiaquick Gel Extraction Kit (Qiagen GmbH) according to manufacturer's instructions. The purified DNA products were then subjected to direct sequencing (Biocenter Oulu DNA Sequencing Core Facility, Oulu, Finland).

4.1.4 Induction of CYP1 mRNAs and expression of CYP1 proteins

To further characterize the induction of CYP1A1 and CYP1B1 by TCDD in human non-pigmented ciliary epithelial cells by an accurate quantitative method, a dose response experiment was carried out and the mRNAs detected with Northern blotting. Total RNA was electrophoretically resolved and transferred onto a Hybond-N^+ nylon membrane (Amersham Biosciences, Little Chalfont, UK). The RNA was fixed by UV-crosslinking, and the membrane hybridized with [α^{32}P] dCTP-labeled CYP1A1 and CYP1B1 cDNA probes.

The presence of CYP1B1 protein was analyzed by Western immunoblotting with human anti-CYP1B1 antibody. The cells were broken by sonication and centrifuged. Protein was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. CYP1B1 Supersomes was used as recombinant control. The proteins were transferred electrophoretically to an Immobilon^{TM} PVDF transfer membrane (Millipore, Bedford, MA, USA). The membrane was blocked overnight. The sheet was then incubated for 1 h with an antibody raised against a human CYP1B1 pentapeptide and for 1 h with a secondary peroxidase-conjugated goat antirabbit antibody. After washing, the immunoreactive bands were visualized with chemiluminescent peroxidase substrate.

4.1.5 Regulatory mechanisms of CYP1B1 induction

To study the regulatory mechanisms of CYP1B1 induction by TCDD in human ciliary cells CYP1B1 5'-luciferase reporter constructs were transfected into the human ciliary epithelial cell line. Ciliary epithelial cells were cultured on a 24-well plate for 24 h before transient transfection using Tfx-20 reagent according to manufacturer's instructions (Abu-Bakar et al. 2007). Cells in each well were transfected with *CYP1B1/luc* reporter gene plasmid and control reporter plasmid pRL-TK in Opti-MEM I medium. Twenty-four hours after transfection the cells were treated with TCDD or vehicle dimethyl sulfoxide (DMSO) only. After 24 h treatment, the luciferase activities were measured by the Dual-Luciferase Reporter Assay System.

4.2 Metabolism and interactions of timolol in human liver homogenate, microsomes and cryopreserved hepatocytes

4.2.1 Chemicals

l-timolol maleate was supplied by Santen Oy (Tampere, Finland). Dextromethorphan, fluconazole, fluoxetine, paroxetine, quinidine and sertraline were purchased from the Sigma Chemical Company (St. Louis, MO, USA). Metabolite standard dextrorphan was obtained from the Ultrafine Chemical Company (Manchester, U.K.). Fluvoxamine was donated by Solvay Pharmaceuticals (Weesp, The Netherlands) and citalopram was from H. Lundbeck A/S (Copenhagen, Denmark). Bupropion, celecoxib, etoricoxib and terbinafine were from Sequoia Research Products Ltd (Pangbourne, UK). Cimetidine was obtained from Smith Kline & French Laboratories Ltd. (Herts, UK).

Other chemicals were mainly from the Sigma Chemical Company and Boehringer (Ingelheim, Germany) and were of the highest purity available. HPLC-grade methanol and acetonitrile were obtained from Merck AG (Darmstadt, Germany). Ammonium acetate, ammonium formate, acetic acid and formic acid were purchased from BDH Laboratory Supplies (Poole, UK). UP-grade (ultra pure, 18.2 MΩ) water was in-house freshly prepared with a Direct-Q (Millipore Oy, Espoo, Finland) purification system.

4.2.2 Human liver preparations

The human liver samples used in the metabolism and interaction studies were obtained from the University Hospital of Oulu as surplus from kidney transplantation donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Finland. Liver homogenates were prepared by homogenizing normal-looking tissue in ice-cold 0.1 mM phosphate buffer, pH 7.4, and the homogenate was used as such in the incubations. Microsomes were prepared from surplus tissue separated by differential centrifugation and the final microsomal pellet was suspended in phosphate buffer (Turpeinen et al. 2004). Baculovirus-expressed human P450s (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5) coexpressing human P450 reductase (Supersomes) were purchased from BD

Biosciences Discovery Labware (Bedford, MA). Human cryopreserved hepatocytes from 10 individual donors were obtained from Celsis *In Vitro* Technologies Inc. (Baltimore, MD, USA).

4.2.3 Metabolism of timolol

To screen the disappearance of timolol and identify the metabolites formed, reactions were induced with human liver homogenate and cryopreserved hepatocytes. Liver homogenenate was incubated in phosphate buffer (pH 7.4, 37°C) with timolol concentrations of 1, 5, and 25 μM. Reactions with nicotinamide adenine dinucleotide phosphate (NADPH) were incubated for 20, 40 and 60 min and reactions without NADPH for 0 and 60 min. Each reaction was terminated by adding ice-cold acetonitrile.

In reactions with cryopreserved hepatocytes timolol solutions diluted in Krebs-Henseleit-bicarbonate (KHB) were added to a 48-well plate in concentrations of 1, 4, 10 and 20 μM and pre-incubated at 37°C in a humified (95%) atmosphere of 5% CO_2 in air. To initiate the reactions hepatocyte suspension was added to the wells in the plate and gently mixed. The plates were incubated at 37°C. Samples were removed at time points 0, 15, 30, 60 and 120 min and ice-cold acetonitrile was mixed with the samples. All incubations were performed in duplicate. The samples were stored at -18°C until analyzed.

Baculovirus-expressed human P450s were used according to manufacturer's instructions. For evaluation of the contribution of different P450s to timolol metabolism, a final concentration of 25 M timolol was used. For kinetic studies with recombinant CYP2C19 and CYP2D6, final concentrations of 0.5, 2, 8, 32, and 128 μM timolol were used.

4.2.4 Evaluation of metabolism-dependent inhibition

The effects of several CYP2C19 and CYP2D6 inhibitors on the metabolism of timolol were studied in human liver microsomes. The basic incubation mixture consisted of the following components: human liver microsomal protein 0.5mg/ml, 25 μM timolol in

water and 1 mM NADPH in an incubation volume of 200 µl. Samples were preincubated for 2 min in a shaking incubator block at 37 ^{o}C and reaction started by adding NADPH. The inhibitors (Table 7) at concentrations of 0 (solvent control), 0.001, 0.01, 0.1, 1 and 10 µM for quinidine and 0 (solvent control), 0.01, 0.1, 1, 10 and 100 µM for all other inhibitors were added to the incubation mixture. All incubations were carried out in duplicate, and incubations were additionally performed without NADPH. Incubation periods of 20 min were used and each reaction was terminated by adding ice-cold acetonitrile with subsequent cooling in an ice-bath. The samples were stored at –18 ^{o}C until analysis.

Table 7. Inhibitors of CYP2C19 and CYP2D6 used in the interaction incubations with microsomes and the enzyme they potently inhibit.

Inhibitor	**Inhibitor of CYP**	
	2C19	**2D6**
Bupropion		X
Celecoxib		X
Cimetidine		X
Citalopram	X	
Etoricoxib		X
Fluconazole	X	
Fluoxetine		X
Fluvoxamine	X	
Paroxetine		X
Quinidine		X
Sertraline		X
Terbinafine		X

Based on the results from the interaction incubations with microsomes, paroxetine, fluoxetine and fluvoxamine were selected for the interaction study with cryopreserved hepatocytes. Incubations in cryopreserved hepatocytes were performed basically following the same protocol as in the disappearence study in these cells. Timolol 4 μM and inhibitor solutions 0.1, 1, 5 and 10 μM for paroxetine and fluoxetine and 1, 10, 50 and 200 μM for fluvoxamine were added to a 48-well plate and pre-incubated at 37°C in a humidified (95%) atmosphere of 5% CO_2 in air. To initiate the reactions hepatocyte suspension (1 x 10^6 viable cells/ml) was added to the wells in the plate and gently mixed. The plates were incubated at 37°C and samples removed after incubation, mixed with acetonitrile and stored at -18°C until analyzed.

4.2.5 Analytical method

Timolol and its metabolites were analyzed using high-performance liquid chromatography – tandem mass spectrometry (HPLC/MS/MS). Samples were thawed at room temperature, shaken, centrifuged and pipetted into vials to await the autosampler run. A Waters Alliance HPLC system (Waters Corporation, Milford, MA, USA) was used in liver homogenate and microsome analyses. Data on the screening of timolol metabolites were recorded with a Micromass LCT time-of-light high-resolution spectrometer (Waters) equipped with a LockSpray electrospray ionization source. Data for interaction studies were recorded with a Micromass Quattro II triple quadropole mass spectometer (Waters) with a Z-ray electrospray ionization source. Samples from hepatocyte incubations were analyzed using a Waters Acquity ultra-performance liquid chromatographic system (Waters). Data were recorded with a Waters LCT Premier XE time-of-light mass spectrometer equipped with a LockSpray electrospray ionization source.

4.2.6 Data analysis

Enzyme inhibition was determined by comparing enzyme activities in the presence of the inibitors with the control incubations (containing solvent but no inhibitor). IC_{50} values were determined for each inhibitor. Values were determined graphically from the plot of the logarithm of inhibitor concentration *vs* percentage of activity remaining after

inhibition using Microcal Origin, version 6.0 (Microcal Software, Inc., Northampton, MA, USA). K_m and V_{max} values were determined graphically from Lineweaver-Burk plots.

4.2.7 *In vitro – in vivo* extrapolations

Pharmacokinetic variables for the metabolism of timolol in the human body were extrapolated from the *in vitro* data obtained from incubations of timolol with liver homogenate and cryopreserved hepatocytes. The intrinsic *in vitro* clearance ($CL_{h,int}$) was first determined and the value obtained used to make an estimate of hepatic clearance (CL_H) and total systemic clearance (CL_{total}) (Pelkonen and Turpeinen 2007). In addition, the half life ($t_{1/2}$) of timolol was calculated.

Calculations were made with following assumptions:

- the number of hepatocytes/g of liver is 120×10^6,
- the mass of the human liver is 1500 g,
- liver blood flow $Q_H = 1.45$ l/min,
- renal clearance (assumption) $CL_R = 0.18$ l/min,
- the mass of the virtual human is 70 kg and
- distribution volume $V_D = 2.5$ l/kg body weight

(Dollery et al. 1991, Davies and Morris 1993, Pelkonen and Turpeinen 2007).

4.3 Absorption of timolol from two ophthalmic preparations into the aqueous humor in humans

4.3.1 Study products

The timolol products Timosan® and Oftan® Timolol used in this study were from Santen Oy, Tampere, Finland.

Timosan®, timolol 0.1% eye gel, contains l-timolol maleate 1.37 mg corresponding to timolol 1 mg, benzalkonium chloride, sorbitol, polyvinyl alcohol, carbomer 974P, sodium acetate, lysine monohydrate and water for injections ad 1 g.

Oftan® Timolol, aqueous 0.5% timolol eye drops contain l-timolol maleate 6.84 mg corresponding to timolol 5 mg, benzalkonium chloride, sodium dihydrogenophosphate dihydrate, disodium phosphate dodecahydrate, sodium hydroxid ad pH 6.8 - 7.0 and water for injections ad 1ml.

4.3.2 Subjects and ethics

Forty-four generally healthy patients scheduled for cataract surgery were enrolled for the study after giving written informed consent. Patients were aged 55-80 years and could be either sex. Patients with any previously set exclusion criteria were not enrolled.

The study protocol was approved by the Pirkanmaa Health Care District Ethics Committee at Tampere University Hospital, Finland. The clinical study was conducted in accordance with current Good Clinical Practice (GCP) requirements and the ethical principles in the Declaration of Helsinki 2004.

4.3.3 Study design

The study was a randomized investigator-masked study. The patients were assigned to receive one of the two study medications timolol 0.1% eye gel or aqueous 0.5% timolol eye drops. The medication was administered into the conjunctival sac of one eye two

hours before the anticipated aqueous humor sample collection. At the beginning of the cataract* operation, an aqueous humor sample was drawn from the anterior chamber. The samples were stored at -20 °C until analyzed.

*Cataract is the leading cause of blindness worldwide (Abouzeid et al. 2009). The disorder is defined as loss of transparency of the crystalline lens of the eye (Bunce et al. 1990, Örnek et al. 2003). The only effective treatment is surgery, the cataractous lens being removed and replaced with an inserted intraocular lens (Olson et al. 2003).

Timolol concentrations were measured from the samples. In order to evaluate the metabolism of timolol in human ocular tissues also the presence and concentration of timolol metabolites was measured.

Using the timolol concentrations obtained in aqueous humor, β-receptor occupancies were calculated to give an estimate of the theoretical efficacy of the products.

Receptor occupancy % = 100 x $(i/K_i) / (i/K_i + 1)$,
where i = timolol concentration in the aqueous humor
K_i = dissociation binding constant

The affinities (K_i-value) of timolol for the rabbit lung β_1-receptor and rat reticulocyte β_2-receptor site have previously been reported as 1.22 nM and 0.42 nM, respectively (Saari et al. 1993).

4.3.4 Analytical method

Timolol concentrations and the presence of metabolites of timolol were analyzed using high-performance liquid chromatography – tandem mass spectrometry. Samples were prepared for analysis by centrifugation, whereafter each sample was spiked with acetonitrile-containing internal standard metoprolol. A Waters Alliance 2695 HPLC system (Waters) was used for analysis. Data were recorded with a Finnigan Surveyor

HPLC instrument with a Finnigan TSQ Quantum Discovery MAX triple quadrupole mass spectometer (San Jose, CA, USA).

4.3.5 Data analysis

Statistical comparison for equality of variances between the groups was made by Levene's robust test statistic with Monte Carlo p-value. Statistical calculations were made using Stata 9.1 software (StataCorp, College Station, TX, USA).

5 RESULTS

5.1 The expression of CYP enzymes in the human ciliary epithelial cells

The mRNAs of the cytochrome P450s studied were all expressed in the human liver, which was used as positive control (Table 8). In human non-pigmented ciliary epithelial cells mRNAs of CYP1A1 and CYP1B1 were detected. The expression of CYP2D6 was further studied as four separate bands were detected (Figure 9). Three of the bands, 1, 2 and 4, were sequenced and confirmed to be parts of the CYP2D6 mRNA. No mRNA expression was detected in any of the negative controls, showing that there was no contamination in the samples.

Table 8. The expression of cytochrome P450 mRNAs in the human liver and ciliary epithelial cells.

CYP enzyme	Liver	Ciliary epithelial cells
1A1	+	+
1A2	+	-
1B1	+	+
2A6	+	-
2B6	+	-
2C8-19	+	-
2D6	+	+
2E1	+	-
3A4	+	-
3A5	+	-
3A7	+	-

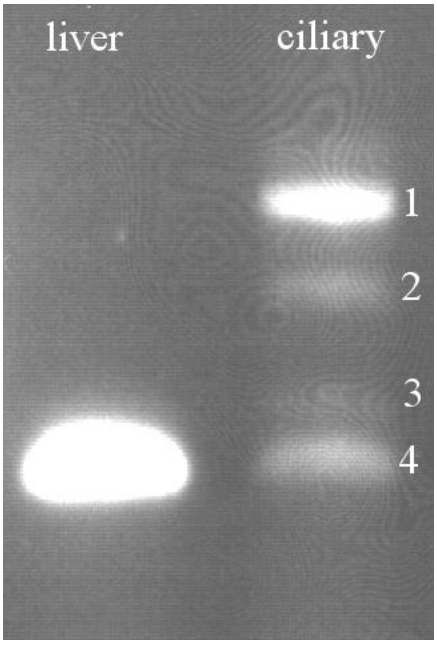

Figure 9. Detection of CYP2D6 mRNA in the human liver and nonpigmented ciliary epithelial cells by RT-PCR.

The RT-PCR method used to study the expression of CYP is not designed to produce quantitative data; however, substantial differences in mRNA amounts can be detected. Expression of CYP1A1 and CYP1B1 mRNAs was strongly induced by 24 h treatment with 10 nM TCDD (Figure 10). TCDD did not induce expression of any other CYP mRNAs. Dexamethasone induced no expression of any of the CYP forms studied.

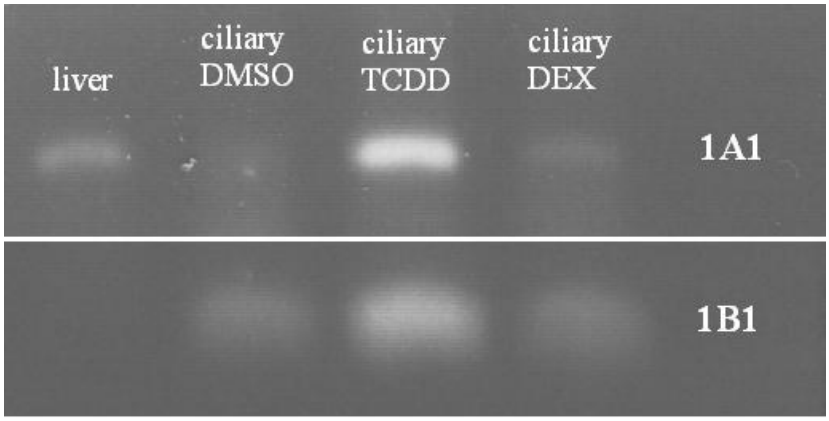

Figure 10. Effect of TCDD and dexamethasone on the expression of CYP1A2 and CYP1B1 in the human nonpigmented ciliary epithelial cells treated with 10 nM TCDD, 100 nM dexamethasone or vehicle (DMSO). The expression was detected by RT-PCR. Liver was used as positive control.

Quantitative analysis using dose-response experiment and Nothern blot analysis showed that the level of CYP1B1 mRNA was increased significantly and dose-dependently by 1 to 100 nM TCDD. The CYP1A1 mRNA was not detected by Northern blot analysis. CYP1B1 protein was expressed in cells treated with TCDD, while no clear protein expression was detected in untreated cells.

5.1.1 CYP-regulating receptor mRNA expression

Expression of mRNAs for the nuclear receptors constitutive androstane receptor (CAR), pregnane X receptor (PXR) and glucocorticoid receptor (GR) as well as the basic helix-loop-helix factors aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) was detected in the liver. In the human ciliary epithelial cell sample GR, AHR and ARNT were expressed, whereas CAR and PXR were not present.

5.1.2 Transcriptional regulation of the *CYP1B1* gene by TCDD

TCDD-induced luciferase activities for *CYP1B1* promoter constructs are presented in Table 9. TCDD induced *CYP1B1* -2299/+25 promoter fragment-regulated luciferase activity 5.7-fold, which indicates transcriptional regulation of the *CYP1B1* gene by TCDD.

Table 9. Effect of TCDD on transcriptional regulation of *CYP1B1*, differences between TCDD-treated samples and DMSO-treated samples. ** $P < 0.01$ vs DMSO treated.

Promoter construct	-fold induction by TCDD
-2299/+25	5.7**
-910/+25	2.4
-852/+25	1.5
-910/XRE3mt	2.0
-910/XRE2mt	2.3

5.2 Metabolism of timolol

5.2.1 Extrapolated pharmacokinetic variables

In human liver homogenate the disappearence of timolol was greatest in incubations with the 1 μM initial concentration, 65% remaining after 20 min. With initial concentrations of 5 and 20 μM approximately 20% of timolol disappeared during a 20 min incubation. The rate of disappearence decreased from 20 to 60 min. In human cryopreserved hepatocytes substrate disappearence was fairly linear at each concentration. The relative substrate disappearence was greatest in incubation with the 1 μM initial concentration, 75% remaining after 120 min incubation. The calculated pharmacokinetic parameters based on the substrate depletion experiments were slightly different in human liver homogenate and in cryopreserved hepatocytes (Table 10).

Table 10. Calculated pharmacokinetic variables for timolol extrapolated from timolol incubations in human liver homogenate and cryopreserved hepatocytes

Pharmacokinetic variable	Liver homogenate	Cryopreserved hepatocytes
CL_{int}	825 ml/min	493 ml/min
CL_H	32 l/h	22 l/h
CL_{total}	42 l/h	33 l/h
K_{el}	0.24/h	0.19/h
$t_{1/2}$	2.9 h	3.7 h

5.2.2 Timolol metabolism

The study with recombinant human CYP enzymed showed timolol to be metabolized mainly by CYP2D6, CYP2C19 making a lesser contribution to the process. In human liver homogenate and microsomes four timolol metabolites were identified, in cryopreserved hepatocytes altogether nine, four the same and five additional. The main metabolite in both systems was a hydroxy metabolite, hereafter called M1. The suggested structures for metabolites are presented in Figure 11. Metabolites M1, M4, M5 and M6 were those formed in the liver homogenate and microsomes.

The K_m values for timolol metabolite M1 formation were 7 μM by CYP2D6 and 50 μM by CYP2C19. In the human liver microsomes the K_m value for M1 was 23.8 μM. The approximate V_{max} value for M1 was 91.2 pmol/min/mg protein in microsomes. For CYP2D6 and CYP2C19 the V_{max} values for M1 were 8.4 and 3.2 pmol/min/pmol, respectively.

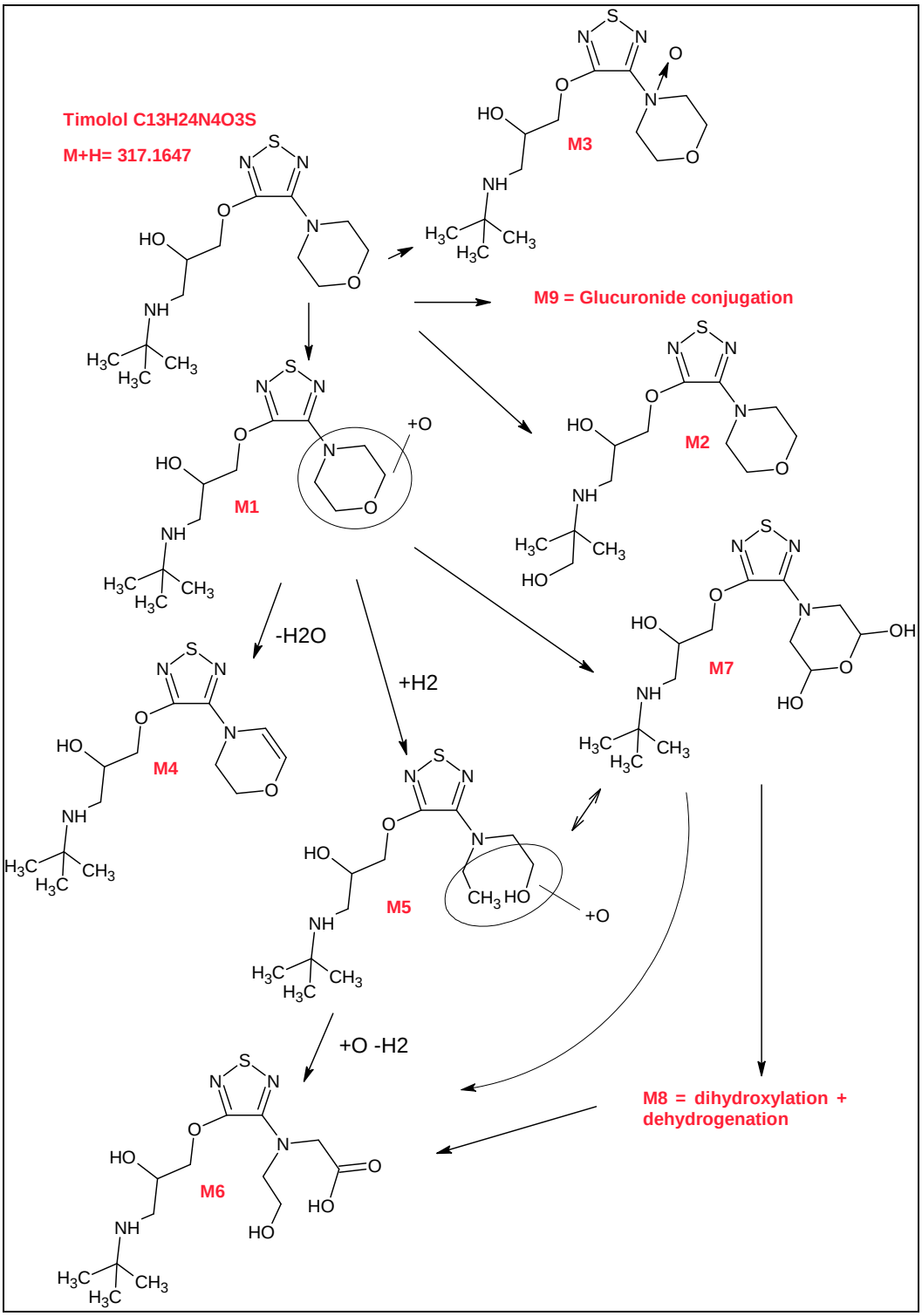

Figure 11. The suggested structures and metabolic routes for the timolol metabolites M1 to M9 detected. The main route is formation of metabolite M1 via hydroxylation; biotransformation takes place in the morpholine ring.

5.3 Inhibition of timolol metabolism

Celecoxib, fluoxetine, paroxetine, sertraline, quinidine and terbinafine were found to be the most potent inhibitors of timolol metabolism in human liver microsomes (Figure 12). They inhibited the formation of timolol metabolites overall with IC_{50} values less than 5 μM. Fluconazole, eterocoxib and cimetidine, on the other hand, showed no inhibitory effect up to 100 μM.

Fluoxetine, fluvoxamine and paroxetine were the inhibitors studied in both human liver microsomes and cryopreserved hepatocytes. Fluoxetine and paroxetine inhibited the formation of all detected timolol metabolites potently. The IC_{50} values for these three inhibitors in the formation of timolol metabolite M1 are presented in Table 11. IC_{50} values were lower in cryopreserved hepatocytes. However, in both systems fluoxetine and paroxetine inhibited the formation of M1 most potently, whereas fluvoxamine was a less potent inhibitor.

Table 11. IC_{50} values (μM) of test inhibitors for the formation of timolol metabolite M1 in human liver homogenates and cryopreserved hepatocytes

Inhibitor	**Human liver homogenate**	**Cryopreserved hepatocytes**
Fluoxetine	1.4	0.7
Fluvoxamine	20.3	5.9
Paroxetine	2.0	0.5

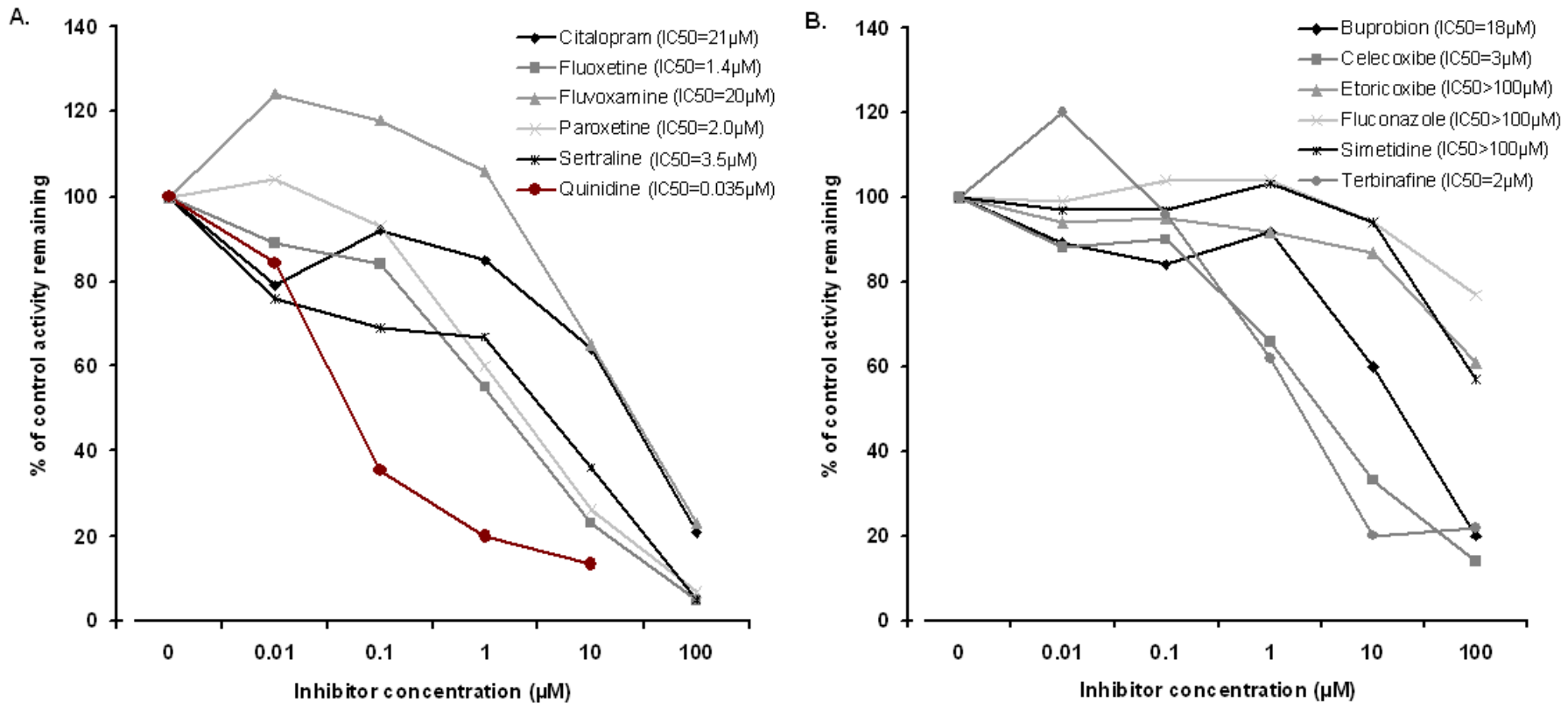

Figure 12. Concentration-dependent inhibition of timolol hydroxymetabolite M1 formation by SSRIs and quinidine (A) and other test inhibitors (B) in human liver microsomes and their IC_{50} values determined using Microcal Origin.

5.4 Timolol and its metabolites in human aqueous humor

Two hours after administration of timolol 0.1% eye gel and aqueous 0.5% timolol eye drops, the concentrations in the aqueous humor were 210 ± 175 ng/ml (mean ± SD) (0.66 ± 0.55 µM) and 538 ± 304 ng/ml (1.7 ± 0.96 µM), respectively. The variation in timolol concentration was much higher after administration of aqueous timolol eye drops ($p = 0.021$) (Figure 13).

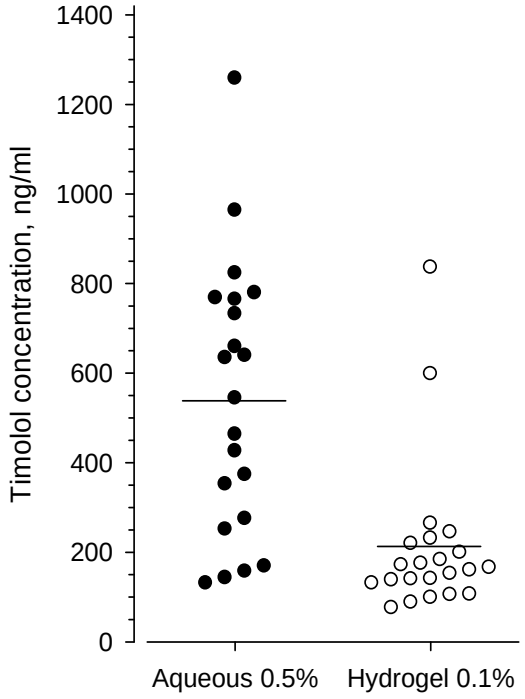

Figure 13. Timolol concentration in aqueous humor of cataract patients two hours after the administration of aqueous 0.5% timolol eye drops (aqueous 0.5%, n=21) or timolol 0.1% eye gel (hydrogel 0.1%, n=22). − mean concentration.

According to the calculated β-receptor occupancies timolol concentrations in the aqueous humor were high enough to occupy virtually 100% of both β_1- and β_2-receptors (Table 12). The receptors were almost 100% occupied even at the lowest concentration determined.

Table 12. Calculated β_1- and β_2-receptor occupancies based on timolol concentration in the aqueous humor.

Product	**Aqueous humor concentration (ng/ml)**		**Molecular concentration (nM)**	**Calculated receptor occupancy (%)**	
				β_1	**β_2**
Timolol 0.1% eye gel	Mean:	210 ng/ml	660 nM	99.8	99.9
	Minimum:	76 ng/ml	240 nM	99.5	99.8
	Maximum:	836 ng/ml	2650 nM	100.0	100.0
Aqueous timolol 0.5% eye drops	Mean:	538 ng/ml	1700 nM	99.9	100.0
	Minimum:	131 ng/ml	410 nM	99.7	99.9
	Maximum:	1258 ng/ml	3980 nM	100.0	100.0

Scarcely any timolol metabolites were present in the human aqueous humor. Very low levels of metabolites were detected in only four persons. In three persons to whom aqueous 0.5% timolol eye drops were administered, concentrations of 16- 25 pg/ml of 3-oxy-metabolite were identified. This 3-oxy-metabolite is one not detected in the *in vitro* experiments. In one person in timolol 0.1% eye gel group less than 10 pg/ml of 3-oxy-metabolite and hydroxyethylglycine metabolite, metabolite M6, was detected.

5.5 The metabolism of timolol in human ocular tissues

Timolol is metabolized mainly by cytochrome P450 enzyme CYP2D6, which was found in *in vitro* experiments. Weak expression of this enzyme was detected at mRNA level in human ciliary epithelial cells. However, only very low levels of timolol metabolites were detected in aqueous humor samples from only four persons in the clinical trial. On this basis no definite conclusions can be drawn as to whether there is CYP2D6 protein present in the ciliary epithelial cells or possibly in some other ocular tissues and whether timolol or other CYP2D6 substrates are metabolized in the ocular tissues. On the other hand, cytochrome P450 enzyme CYP1B1 was detected in the ciliary epithelial cells at protein level. Substrates of CYP1B1 enzyme could be metabolized in the human ciliary epithelium if this functional enzyme is present in the human eye.

6 DISCUSSION

6.1 Methodological aspects

6.1.1 *In vitro* studies

The expression of CYP enzymes in human ocular tissue was studied using a nonpigmented human ciliary epithelial cell line. During passages of the cells some changes e.g. in the expression of enzymes may possibly take place. Therefore, in order to obtain a precise picture of the real *in vivo* situation in the human eye direct analysis of human tissue should be made. However, for practical and ethical reasons the availability of human ocular tissues is very limited. On the other hand, the cell line also has advantages. It represents a pure cell population, while tissue is always a mixture of different cell types. Furthermore, the regulatory aspect of human cells can only be studied in cultured cells. The present cell line is one of the most extensively studied of those derived from the human ciliary epithelium, in part because it maintains *in vitro* some differentiated functions reported to exist *in vivo* (Escribano et al. 1994).

Drug metabolism and interaction studies in human liver preparations give only an estimate of the situation in humans. Exact predictions based on the results from these *in vitro* experiments do not necessarily correlate with *in vivo* findings in humans. However, the human liver homogenate, liver microsomes and cryopreserved hepatocytes used in the metabolism and interaction studies are prepared from the human liver. These tissues are thus the best material for *in vitro* studies when drug metabolism in humans is the real focus of interest. Even though *in vitro* studies do not take into account the situation in which other tissues in addition to the material studied have an effect on the drug in the whole human body, they nonetheless give precise information of that specific liver material studied. *In vitro – in vivo* extrapolations are generally considered useful and the most precise tool for predicting the pharmacokinetics of a drug (Pelkonen and Turpeinen 2007). *In vivo* studies without information on possible risks for a human are not even ethically justified. Preliminary estimates of drug metabolism and possible interactions can be made based on *in vitro* experiments when these aspects are kept in mind. In the present study three different liver preparates were

used, which enhances the reliability of the findings. Further clinical studies in humans can be evolved using information from tissue experiments as a basis for study design.

The microsomes and hepatocytes used in *in vivo* experiments both comprised a pool from ten individual donors. These pooled preparations gave a picture of average metabolism activities, not the situation in one individual. Pooled preparations were selected as there may be wide variations between individuals and selecting e.g. a poor or extensive metabolizer would give an estimate for only that phenotype. As there is no "standard" metabolizer, it was considered appropriate in these initial studies to assess the average metabolism of timolol. The metabolism and interactions of timolol could be further studied in different individual metabolizers in order to establish whether there are marked differences between individuals (Pelkonen and Turpeinen 2007).

In making *in vitro – in vivo* extrapolations some basic hypothesis such as the mass of human liver, liver blood flow, renal clearance and the mass of the human were used. These assumptions are widely used in calculations (Dollery et al. 1991, Davies and Morris 1993, Pelkonen and Turpeinen 2007). Nevertheless, one could say that these values do not necessarily correlate with an individual human subject. Considering the *in vivo* situation the results from clinical trials are likewise an average of the subjects participating in the trial. Inclusion and exclusion criterias make participants at least in some aspects a selected population, but there may also be quite wide variations in these groups. Extrapolations can thus also be considered as average situation, in which exceptions are possible.

6.1.2 *In vivo* study

The clinical trial was conducted as a single-masked, investigator-masked, study. The study nurse administered the product to the patients, and it was not specifically revealed which one of the products was administered. Using placebos in an ophthalmic absorption study would have influenced the absorption of the active ingredients from the study product and placebo would have washed out some of the previously applied product and reduced the amount of active ingredient possibly absorbed (Fechtner and

Realini 2004). The primary end point was not affected even though the study subject or even the investigator had known the product administered.

Timolol concentrations in the aqueous humor were measured two hours after administration of the study products. The time-point was selected based on the manufacturer's rabbit studies, being assumed to be close to the time of maximum concentration in the aqueous humor. However, there are differences in absorption of effective agents from different ophthalmic products and between individuals. For practical reasons this was nonetheless the only way to conduct the study, as it is extremely difficult to obtain an entire concentration-time curve for timolol concentrations in the aqueous humor. In order to obtain a real concentration time curve, samples would need to be from one subject at several time points, which is not possible. For this reason measuring aqueous humor samples at one time point is a generally accepted method in ophthalmic absorption studies. The notable advantage of this kind of study is that the sample is taken during planned ophthalmic surgery, which makes for relatively easy sampling.

6.2 Cytochrome P450 enzymes in human ciliary epithelial cells

A topically applied drug in the treatment of glaucoma enters the aqueous humor through the cornea. However, there has been only limited information on the presence of drug-metabolizing CYP enzymes in the human eye. In the human ciliary epithelial cell line in the present study, mRNA expressions of CYP1A1, CYP1B1 and CYP2D6 were detected, while most of the drug-metabolizing CYP enzymes were not expressed. The expression of CYP2D6 mRNA was however weak, and a few other splice variants were also detected. Thus no definite conclusions concerning CYP2D6 in this ocular tissue can be drawn.

In contrast, CYP1A1 and CYP1B1 mRNAs were strongly expressed in the ciliary epithelial cell line. Previously CYP1B1 has been detected at mRNA level in some ocular tissues and CYP1B1 protein has been shown to be expressed in the fetal and

adult nonpigmented ciliary epithelium (Stoilov et al. 1998, Doshi et al. 2006). The present results are in line with those previously reported. However, for the first time we showed marked induction of both CYP1B1 mRNA and protein by TCDD in the ciliary epithelial cells. TCDD induction of CYP1B1 is suggested to be mediated by AHR, one of the receptors which controls the induction of CYP1 family members (Hakkola et al. 1997). TCDD, which is known to be a toxic compound, is a potent AHR ligand; AHR mediates the cellular response to TCDD (Chiaro et al. 2007). CYP1B1 has been associated with congenital as well as primary open-angle glaucoma. However, its role is not fully understood (WuDunn 2002, Vasilou and Gonzalez 2008). It has been suggested that CYP1B1 participates in the development of the iridocorneal angle (Lopez-Garrido et al. 2006). Whether normal CYP1B1 regulation can be disturbed e.g. by exposure to exogenous AHR ligands and whether this could lead to the development of glaucoma during fetal development or in later life remains an open question which warrants further investigation.

6.3 Metabolism of timolol

Though timolol has been in clinical use for decades, little information has been available on its metabolism. In the present study, in human liver homogenate and microsomes four metabolites (M1, M4, M5 and M6) were formed, while in cryopreserved hepatocytes altogether nine metabolites could be detected. One disadvantage in the use of microsomes is the loss of enzyme activity during preparation (Pelkonen and Turpeinen 2008). Hepatocytes, again, are known to contain a full scale of both phase I and II metabolizing enzymes. This could be one reason for the difference in numbers of metabolites. On the other hand, there were some differences in incubation periods between experiments due to the different durations of enzyme activities in liver preparations.

The previously suggested metabolism of timolol (Edeki et al. 1995, Ishii et al. 2000) by CYP2D6 was confirmed in this study. CYP2C19 was also found to make a contribution to timolol metabolism, albeit to a lesser degree. The clinical role of CYP2C19 is not

clear, but in theory it could be of significance in timolol metabolism in CYP2D6 poor metabolizers. The timolol plasma concentration, which would be otherwise increased as compared to an extensive metabolizer, could be "normalized" by metabolism through the CYP2C19 enzyme. However, the situation is not so clear, as CYP2C19 is also a polymorphically expressed enzyme. The effect would be different in a person having both poor CYP2C19 and CYP2D6 phenotype as compared with a person having some other combination of enzyme phenotypes.

The extrapolated pharmacokinetic variables, total body clearance and half-life correlated fairly well with those previously reported in humans (Table 13). Mäntylä and co-workers (1983) reported a total body clearance of 751.5 ± 90.6 ml/min (45 ± 5.4 l/h) in healthy volunteers. Differences in clearance between individuals were detected. Extrapolated clearances from liver homogenate and cryopreserved hepatocytes can therefore be assumed to approximate fairly well to the average clearance in humans. Based on these results liver preparations may be considered suitable for studying timolol metabolism *in vitro*.

Table 13. Pharmacokinetic parameters for timolol extrapolated from human liver homogenate and cryopreserved hepatocyte incubations and reported for human subjects.

Pharmacokinetic parameter	**Liver homogenate**	**Cryopreserved hepatocytes**	**Human**
CL_{total}	42 l/h	33 l/h	45 ± 5.4 l/h[1]
$t_{1/2}$	2.9 h	3.7 h	2 – 5 h[2]

[1]Mäntylä et al. 1983, [2] Dollery et al. 1991.

6.4 Timolol drug interactions

The CYP2D6 inhibitors celecoxib, fluoxetine, paroxetine, sertraline, quinidine and terbinafine were found to be potent inhibitors of timolol metabolism in human liver microsomes. Fluoxetine and paroxetine were also potent inhibitors in cryopreserved hepatocytes. Pharmacokinetic variables extrapolated from liver preparations correlated fairly well with those previously reported in humans. It may thus be assumed that the marked interactions detected in microsomes and cryopreserved hepatocytes could also be of importance in clinical use. Glaucoma patients are mostly elderly persons, who may be on one or more drugs concomitant with an ophthalmic timolol product. Since ophthalmic preparations are administered topically, the possibility may well be overlooked that they may cause clinically significant adverse systemic effects or drug-drug interactions. The fact is that ophthalmic timolol has been shown to be systemically absorbed and to cause systemic adverse effects (Nelson et al. 1986, Spratt et al. 2006). The results of the present study suggest that especially fluoxetine and paroxetine may increase the plasma concentrations of timolol in man. This should be taken into consideration for example when prescribing antidepressive treatment to glaucoma patient.

Patients using glaucoma medications may have different CYP2D6 phenotypes, which affects the plasma levels reached after timolol administration. High plasma timolol concentrations may be related to the CYP2D6 PM phenotype or a concomitantly administered CYP2D6 inhibitor. It has been suggested that the possibility of cardiac effects increases when the plasma level of timolol is over 0.7 μg/l (2.2 nM) (Nieminen et al. 2005).

Aqueous humor concentrations in the present study were a hundred times higher than the above-mentioned plasma concentration suggested as “cut-off” value for cardiac effects. After administration of 0.1% timolol eye gel plasma levels of about 0.2 μg/l have been reported (Uusitalo et al. 2005, Uusitalo et al. 2006). Even if a large proportion of the administered dose of timolol 0.1% eye gel were absorbed into the systemic circulation it would not reach concentrations sufficient to cause cardiac effects.

Either the poor-metabolizer phenotype or use of a concomintant CYP2D6 inhibitor should not cause an increase in plasma concentrations to adverse levels.

6.5 Metabolism of timolol in the human eye

Very low levels of two timolol metabolites were detected in the human aqueous humor samples of only four subjects to whom a timolol ophthalmic product was administered two hours before a cataract operation. The metabolite M6 was the same as that detected in experiments conducted with human liver preparations. The other metabolite was 3-oxy-metabolite, which was not detected in *in vitro* experiments but has previously been detected as an *in vivo* metabolite in the dog and rat (Tocco et al. 1980). It is plausible that these metabolites were formed in the ocular tissues and not in the liver. Several CYP2D6 mRNA splice variants were detected in the human ciliary epithelial cells. However, the amount of CYP2D6 mRNA was very low. It would appear that a negligible amount of CYP2D6 protein is expressed in the human ocular tissues.

6.6 Timolol concentrations in human aqueous humor

It was shown here that the formulation of an ophthalmic product is an important factor in drug absorption. The inter-individual variation after administration of timolol 0.1% eye gel was only slight. On the other hand, after aqueous 0.5% timolol eye drops the variation was much higher, as has also been shown in previous studies measuring aqueous humor concentrations after administration of aqueous timolol eye drops (Blanksma 1991; Saari et al. 1993). The aqueous humor concentrations were lower after administration of timolol 0.1% eye gel, which was to be expected, as the timolol concentration in the product is only one fifth of that in the 0.5% eye drops. However, this lower concentration was calculated to occupy practically 100% of both β_1- and β_2-receptors. It has previously been shown that the IOP-lowering effect is comparable between timolol 0.1% eye gel administered once daily and aqueous 0.5% timolol eye

drops applied twice daily (Rouland et al. 2002). The β_1- and β_2-receptors were calculated to be occupied even at the lowest detected concentration, 76 ng/ml. Timolol has been on the market for over three decades and its safety profile is well known. As discussed in section 2.4.4, topically applied timolol may have adverse systemic e.g. cardiac and pulmonary effects. The lowest possible timolol concentration in the aqueous humor is thus desirable. Long-term topical use of glaucoma treatment has been suggested to increase the risk of cataract (Leske et al. 2002, Chandrasekaran et al. 2006, Herman et al 2006), and from this point of view low aqueous humor levels are likewise valuable. Physicians should carefully consider whether 0.5% eye drops or 0.1% eye gel is prescribed as IOP-lowering drug. When an appropriate product with low timolol concentration is selected, intraocular safety can possibly be increased and the risk of adverse systemic effects can be minimized with nonetheless adequate treatment effect.

7 SUMMARY AND CONCLUSIONS

The aim of the present study was to establish the presence of cytochrome P450 enzymes in the human ocular tissue, the metabolism and interactions of timolol and the absorption of timolol into the aqueous humor from two different commercially available timolol products widely used in the treatment of glaucoma.

The main findings were:

- In human ciliary epithelial cells, participating in aqueous humor formation, CYP1A1 and CYP1B1 mRNA were expressed. Both CYP1B1 mRNA and protein expression were strongly induced by TCDD. CYP1B1 induction is suggested to be mediated by AHR.

- Low levels of CYP2D6 mRNA splice variants were expressed in the human ciliary epithelial cells and very low levels of timolol metabolites were detected in the human aqueous humor. It seems that a negligible amount of CYP2D6 protein is expressed in the human ocular tissues.

- Timolol is metabolized mainly by CYP2D6, CYP2C19 having a minor role. The main metabolite of the nine identified was a hydroxyl metabolite, M1.

- CYP2D6 inhibitors, especially fluoxetine and paroxetine, are potent inhibitors of timolol metabolism. This inhibition may be of clinical significance in patients using ophthalmic timolol products.

- Timolol 0.1% eye gel leads to aqueous humor concentrations high enough to achieve total β_1- and β_2- receptor occupancy, i.e. therapeutic effect. Inter-individual variation in concentrations with timolol 0.1% eye gel was shown to be low. Intraocular and systemic safety can be increased when this product with low timolol concentration is used.

In conclusion, the drug-metabolizing cytochrome P450 enzymes CYP1A1, CYP1B1 and CYP2D6 are expressed in the human ciliary epithelial cells at mRNA level and CYP1B1 also at protein level. Low concentrations of timolol metabolites were detected in the human aqueous humor after ophthalmic administration of timolol, which indicates some CYP2D6 activity present in the human ocular tissues. Timolol 0.1% eye gel proved a good treatment option for a glaucoma patient in respect of differences in ophthalmic absorption and metabolic properties between individuals and possible interactions with concomitant CYP2D6 inhibitors.

8 ACKNOWLEDGEMENTS

This work was carried out at the Institute of Biomedicine, Pharmacology in the University of Helsinki, at the Department of Pharmacology and Toxicology in the University of Oulu, the Department of Eye, Ear and Oral Diseases in Tampere University Hospital and at Santen Oy, Tampere. I am grateful to all those who have helped me during this undertaking.

I have been privileged to be supervised by Professor Heikki Vapaatalo, whose fruitful discussions, advice and encouragement have taken this thesis forward. I am grateful to have learnt to know him. I sincerely thank my other supervisor Docent Jukka Mäenpää for all his support during these years. The contribution of my third supervisor Professor Jukka Hakkola in introducing me to the fascinating field of cytochrome P450-enzymes is gratefully acknowledged. It was always pleasant to visit Oulu. It has been a pleasure to be guided to the field of research by such experts.

I wish to express my gratitude to Santen Oy, the Vice-President of Research and Development Kari Lehmussaari and Director of Clinical Research and Medical Affairs Auli Ropo for providing possibilities to do this work. I also acknowledge my debt to Professor of Pharmacology Esa Korpi, Head of the Institute of Biomedicine in the University of Helsinki, for making this thesis possible. Professor Olavi Pelkonen Head of the Department of Pharmacology and Toxicology in the University of Oulu is deeply thanked for giving me the opportunity to do the experiments in Oulu.

I wish to thank all my co-authors for their contributions. I express my deepest gratitude to Docent Miia Turpeinen for guiding me through the first *in vitro* experiments and for all her support and friendliness. I most sincerely thank Dosent Ari Tolonen and Jouko Uusitalo, M.Sc. for all the work they have done in measuring drug concentrations in both the *in vitro* and *in vivo* studies. Timo Korjamo Ph.D. is warmly thanked for help and co-work during the hepatocyte experiments. I am most grateful to Professor Esko Aine for collaboration during the *in vivo* study. The statistical knowledge and advice of Hannu Kautiainen, B.A. is greatly appreciated. I am thankful to Esko Kankuri M.D.,

Ph.D. and Olli Oksala Ph.D. for their expertise and comments. Without you all, this thesis would have not been possible.

Miki Nakajima and Tsuyoshi Yokoi from Kanazawa University are thanked for providing *CYP1B1* promoter constructs and Professor Miguel Coca-Prados from Yale University for providing ciliary epithelial cells.

I highly appreciate the kind and skilful help of Ritva Tauriainen and Päivi Tyni in carrying out the laboratory work. I express my gratitude to Anne Pielismaa for her contribution in the clinical trial.

I owe a debt of gratitude to Kalle Oinas for all his valuable help with computer-related issues.

Professor Kai Kaarniranta and Docent Jari Lilja are sincerely thanked for reviewing my thesis manuscript and for the constructive criticism and comments. Robert MacGilleon is thanked for checking and correcting the English language.

The work was financially supported by Santen Oy, the Finnish Funding Agency for Technology and Innovation and the Academy of Finland, which support is gratefully acknowledged.

I am grateful to my friends for all their support and discussions - for sharing all kinds of feelings during this process. Without you this task would have been much harder. I hope these general words are sufficient to express my gratitude to each of you.

Finally, I thank my parents, Anja and Eino, my sister Matleena and my brother Ilpo for all their precious support and encouragement. Thank you for being there whenever needed.

Tampere, December 2009
Marjo Volotinen

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