Examination of the penetration behavior of pharmaceutical drugs on the eye and set-up of an in vitro cell model for ocular drug delivery

Dissertation zur Erlangung des Grades

des Doktors der Naturwissenschaften

der Naturwissenschaftlich-Technischen Fakultät III

Chemie, Pharmazie, Bio- und Werkstoffwissenschaften

der Universität des Saarlandes

von

Ulrich Becker

Saarbrücken

2006

Tag des Kolloquiums:	05.Dezember 2006
Dekan:	Prof. Dr. Kaspar Hegetschweiler
Mitglieder des Prüfungsausschusses:	Prof. Dr. Alexandra Kiemer
	Prof. Dr. Claus-Michael Lehr
	Prof. Dr. Hans H. Maurer
	Dr. Carsten Ehrhardt
	Dr. Marc Schneider

TABLE OF CONTENTS

Introduction

The eye as target for topical drug delivery – overcoming the problems

Anatomy

Anatomy of the eye

The eye is the organ for visual perception of the environment. In order to forming a picture, a perceptive and a refractive system is needed. The perceptive system is located in the rear part of the eye, while the refractive system is mainly located in the anterior part.

Figure 1 gives an overview on the architecture of the eye.

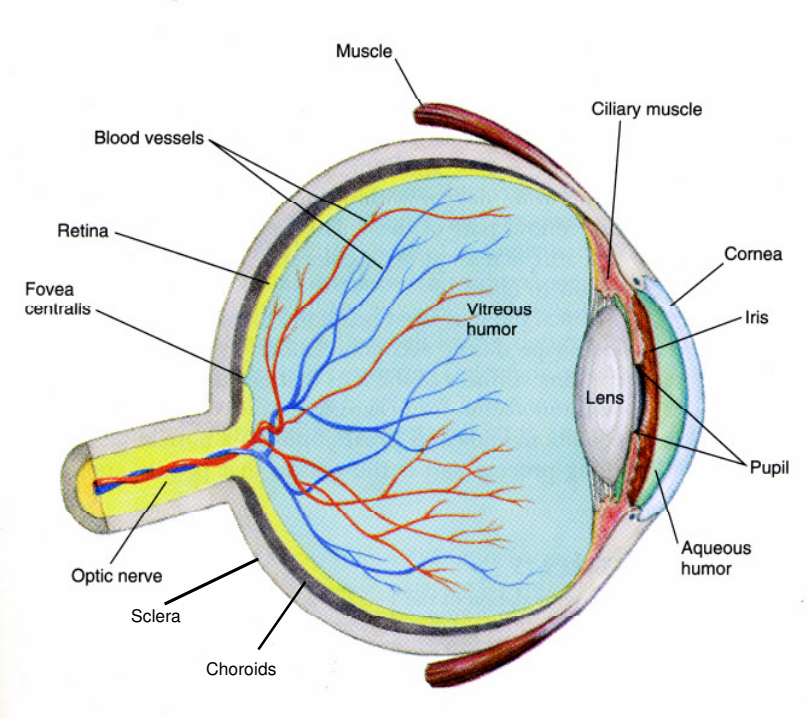


Figure 1: *Anatomy of the eye [adapted from ref. 1]*

The shape of the eyeball is crucial for the proper function of the eye. Therefore, its shape sustained by a system composed of an outer shell that is build of various layers of tissue and an additional inner support, the vitreous body. The outmost tissue layer of the eye is the *tunica externa oculi* and it mainly consists of connective tissue, called

sclera. The sclera has a white appearance and is made of collagen. The front part of this outer shell is the cornea, which is incremented to the sclera in an hourglass-like fashion. The cornea is clearly distinguishable from the sclera and has different properties, of which the optical (refractive) and barrier function are the most important ones.

The middle layer of the eye is called uvea and consists of choroids, iris and ciliary body. The choroid contains a big amount of blood vessels and is responsible for the nutrition (i.e., supply with oxygen, minerals and other nutrients) of the adjacent tissues. Connected to the choroids is the ciliary body, which is responsible for the accommodation of the lens. It also regulates the intraocular pressure by disposing the aqueous humor through the Schlemm's channel. Third part of the uvea is the iris. It consists of two important smooth muscles, called musculus sphincter pupillae and musculus dilatator pupillae. These two muscles control the opening and closing of the pupilla, depending on light intensity on the retina.
Furthermore, the iris has a pigmented epithelium that determines its color. The epithelium is visible from the outside and is a personal feature, commonly known as eye color. Besides the control of light intensity entering the eye and giving the eye color, the iris serves as a barrier between the anterior and the posterior part of the eye. Thus, it creates the anterior chamber and posterior chamber that contain the aqueous humor.

The inner parts of the eye consist of anterior chamber, lens, vitreous body and retina. The retina is the innermost layer of the bulbus and is the perceptive system of the eye. Through photochemical processes in the light sensitive cells (i.e., rods and cones)

nervous impulses are generated that will be transformed to a picture in the brain. Through the high density on pigmented cells, the retina has a dark-brown to black appearance.

The vitreous body mainly helps maintain the spherical shape of the eyeball.

The lens is, besides cornea, the second part of the refractive system that directs light to the perceptive part of the retina. The curvature of the lens is controlled by the ciliary body.

The anterior chamber is the liquid-filled part between cornea and iris. It is mainly responsible for the intraocular pressure of 10-21 mm Hg.

Anatomy of the cornea

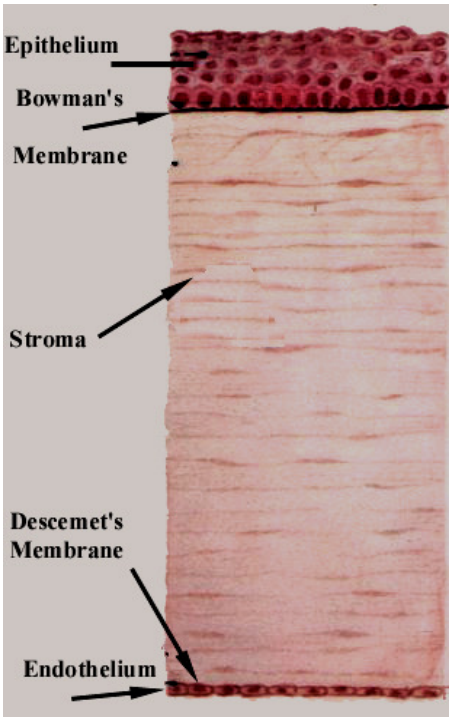


Figure 2: *The layers of the corneal epithelium [taken from ref. 2]*

The cornea has two main characteristics. On the one hand, it is part of the refractive system and directs, together with the lens, light towards the macula lutea on the retina.

To serve this property, it is necessary that the cornea has a clear appearance. This is achieved by a high degree of hydratization and the absence of blood vessels [5]. Nutrients are delivered to the cornea by diffusion only. On the other hand, it represents the barrier between the anterior chamber and the air in the outside environment. This makes the cornea an important part of the protective system of the body, because it prevents substances and microorganisms from entering the eye. Histologically, the structure of the cornea is rather simple. As shown in figure 2, it consists of a multi-layered epithelium, two membranes, a stroma and a single-layered endothelium.

The human epithelium itself consists of 5-6 cell layers, but the thickness varies between species and can easily exceed the numbers found for humans. The innermost layer is the basal cell layer. This monolayer contains living cells with mitotic activity. Cells are produced continuously. During their life span of about 14 days, epithelial cells differentiate and travel to the surface of the epithelium, becoming first wing cells (2-3 cell layers) and then superficial cells with a very long and flat appearance. The superficial cells completely lack proliferative activity [6]. The surface of the cornea is highly increased by mircoplicae and microvilli. The epithelial layers also contain a high number of nervous endings that produce the feeling of major discomfort if the eye is rubbed or scratched. Tight junctions between neighboring cells of the epithelium are essential for the protective properties of the cornea and also for the maintenance of corneal transparency [3, 4]

Basis for the epithelium is Bowman’s membrane. It mainly consists of type I and III collagenous fibers. The actual purpose of this membrane is still unclear, especially

because not all mammals have a Bowman's membrane. But in humans, the importance of this layer for structural integrity of the epithelium has been shown. The body does not have self-repair mechanisms for the Bowman membrane [7].

Almost 90% of the cornea consists of stroma. This middle layer consists of water and collagen type I (90%), III (<10%), and V (traces) [8]. The collagen is produced by fibroblasts that are embedded in the stroma. The organization of the collagen fibrils is crucial for the transparency of the cornea.

Descement's membrane serves as basal layer for the endothelium. It consists of collagen type IV, fibronectin and laminin [8].

The endothelium is the innermost layer of the cornea and the barrier to the aqueous humor. It controls substance diffusion and nutrition of the upper layers. It is a tight layer through formation of cell-cell-contacts, but not the major barrier in the cornea. The normal density is about 3,500 endothelial cells/mm^2.

Targets for ocular drug delivery

The treatment of ocular diseases is in general pursued by local, topical application of medical drugs. Carriers for the drugs are usually eye drops or eye ointments.
For the drug, the hydrophilicity/lipophilicity ratio determines the penetration of the substance. Hydrophilic substances will stay on the surface of the eye, accumulating in the tear film. Highly lipophilic substances will penetrate the corneal epithelium but are unable to cross the hydrophilic stroma. For these substances, other routes of

penetration, like distribution through the local vasculature, are reserved [9].
Amphiphilic substances do have the properties to cross the various corneal compartments with different characteristics (i.e., lipophilic or hydrophilic corresponding to corneal epithelium and endothelium or stroma) and penetrate into the inner eye.

In the eye, there are application possibilities for both, hydrophilic and lipophilic drugs. Pharmaceutical treatment of the eye surface includes (allergic) conjunctivitis, dry eye disease, bacterial infections of the eyelid limbus (i.e., hordeolum), local anesthesia and cornea ulcers.
Pathological conditions of the inner eye are glaucoma, age-related macula degeneration (AMD), eye infections with bacteria, viruses and fungi, cataract and retinal detachment.
This makes aqueous humor/ciliary body, lens and retina to major targets for ocular drug delivery in the inner eye.

For an amphiphilic substance, there are 3 general ways of drug delivery. The first possibility is the general blood circulation that will distribute the substance in the systemic circulation and, following drug distribution in the body, also reach the eye locally.
Another pathway is the penetration via the conjunctival-scleral pathway that includes distribution of the substance along the conjunctiva and re-entry of the compound into the eye through the sclera [9]. The conjunctival-scleral pathway is restricted to some compounds of high molecular weight. Even though it contributes highly to the drug delivery of these compounds, it is of minor interest for general ocular drug delivery.

The most common way of drug penetration is the direct pathway through the cornea, which makes this compartment of the eye a major barrier for ocular drug delivery. Drug penetration in the cornea has two possible pathways: crossing the cellular body (i.e., transcellular) or through the gaps between the cells (i.e., paracellular). For paracellular transport, tight junctions between the uppermost layers of the superficial cells form a major barrier for penetration [10]. More hydrophilic drugs can only cross the barrier by using pores in the cell layer. The tight junctions regulate the 6nm-sized openings. More lipophilic drugs have the properties for transcellular penetration. The lipophilicity ratio has been shown to be a crucial factor for transcellular transport by Schoenwald and others [11]. The different pathways through cellular layers are shown in Figure 3. The most important ways of drug penetration in corneal tissue are shown on the left hand side.

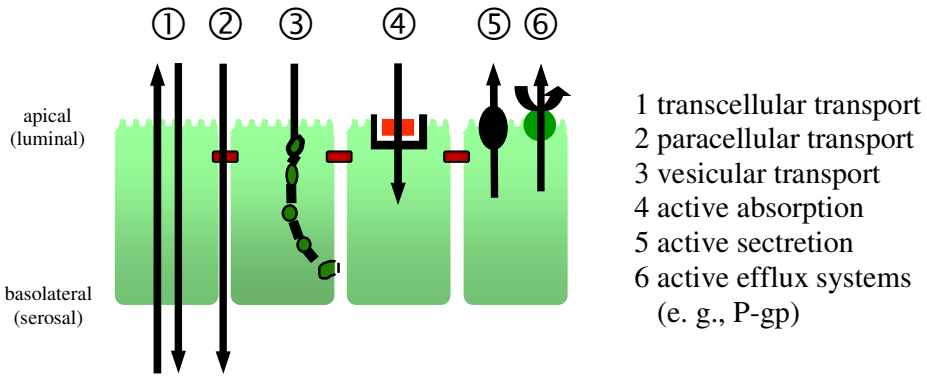


Figure 3: *Penetration pathways in (cultured) cell layers*

Besides the physico-chemical properties of the active ingredient, several other aspects affect the delivery of drugs to the eye. As an important factor, the short residence time for ocularly applied solutions has to be considered. This is caused by the small total volume of the cul-de-sac and rapid drainage of the liquid through the nasal duct. Thus,

for an ocular drug preparation, viscosity, mucoadhesive properties, applied volume and moistening are crucial factors.

In vitro models to study drug absorption

Drug delivery research has gained a new tool in the last decades: many pharmaceutical scientists use *in vitro* cell cultures as novel strategy to exploit drug delivery issues. The various advantages of *in vitro* cell cultures in pharmaceutical development and research include (1) rapid assessment of the potential permeability and metabolism of a drug, (2) the opportunity to elucidate the molecular mechanisms of drug transport or the pathways of drug degradation (or activation), (3) rapid evaluation of strategies for achieving drug targeting, enhancing drug transport, and minimizing drug metabolism, (4) the opportunity to use human rather than animal tissues, (5) the opportunity to minimize time consuming, expensive, and sometimes controversial animal studies. The reduction of animal experiments is in accordance with the 3 R principles, firstly introduced by Russell and Burch in 1959 [12]. The 3 R's stand for **R**eplace, **R**educe and **R**efine and are guidelines for the application of animal experiments in scientific research. In a sense of animal welfare, cost reduction and a wish for increased reproducibility of experiments, the replacement of animal experiments by suitable *in vitro* test systems has become important in the last decades. In an effort to pursue this scope, an array of cell culture models for epithelial barriers has been established, including models of the intestinal, rectal, buccal, sublingual, nasal, ocular epithelium and the epidermis of the skin [20]. Especially, the epidermal cell models (i.e., reconstituted epidermis) and the intestinal epithelium (i.e., Caco-2 cell cultures) have gained major acknowledgement in scientific research. But also the

cell culture models of the ocular barriers provide powerful systems to investigate the barrier properties of the eye *in vitro*.

Generally, two types of cell culture systems have to be distinguished: primary cell culture and continuous (immortalized) cell lines.

Primary cultures are derived from cells, tissue or organs that were explanted directly from the donor organism. After purification and selection steps, the cells are seeded onto suitable supports and cultured throughout their lifecycle. No steps for extending the useful lifespan are undertaken. Generally, subculturing of primary cells is possible, but the number of passages is limited due to the limited lifespan.

The limited viability of the cells is seen as a major disadvantage of the primary culture, along with limited availability of the original tissue. The high resemblance of the cells to the original tissue or organism is considered advantageous in return.

Continuous cell lines, also often referred to as immortalized cell lines, have undergone a procedure to extend their useful lifespan beyond their physiological lifecycle. Immortalization often includes the insertion of additional genomic information to the cell and goes along with a decrease in the status of differentiation.

The advantages of continuous cell lines include the uniform characteristics, almost unlimited self-reproduction of the cells and easy handling. Disadvantages of these models include the lower state of differentiation when compared to primary cells and resulting from this, the reduced resemblance to the original tissue or organism. Figure 4 shows a general example of cells cultured on a suitable, permeable filter membrane.

A selection of continuous cell lines representing the corneal epithelium has already been established [13, 14, 14, 16, 17]. Since all these cell lines have undergone a process to extend their lifespan and are partly of non-human origin, they often lack certain features that are only found in human primary cells. Among these features is

epithelial phenotype, state of differentiation, formation of tight junctions, morphological appearance, and presence or absence of membrane transporters. Primary cell cultures are expected to develop a tight barrier in culture and resemble the *in vivo* barrier best by a process of formation of a tight cell layer, differentiation, morphological and functional resemblance. Primary cultures are gained by isolation of the respective cells from the respective (here: corneal) tissue. The fresh cells are maintained in a suitable cell culture medium for a certain time. Since primary cells are in the regular cell cycle, their lifespan is not extended and these cells are mortal. Primary ocular cell cultures, suitable for drug transport studies, have already been established using rabbit corneal epithelial and conjunctival epithelial cells [18, 19]. Taking in account the principles and techniques mentioned above, a wide array of useful cell culture models of the corneal epithelium has been established. The ban of animal testing in the cosmetics industry by the European Union in 2003 contributed to the immense development and improvement of (ocular) cell cultures. Major target was to substitute animal experiments by suitable *in vitro* methods. Many of these cell culture models focus on toxicity testing and ocular irritation, but also cell layers for drug permeation studies are available. Indispensable for successful drug penetration testing is a cell layer that exhibits a tight epithelial barrier, which disqualifies some of the models of the corneal epithelium that were established as substitute for the Draize test. Still, at least two cell lines are available for pharmaceutical purposes (see later chapters).

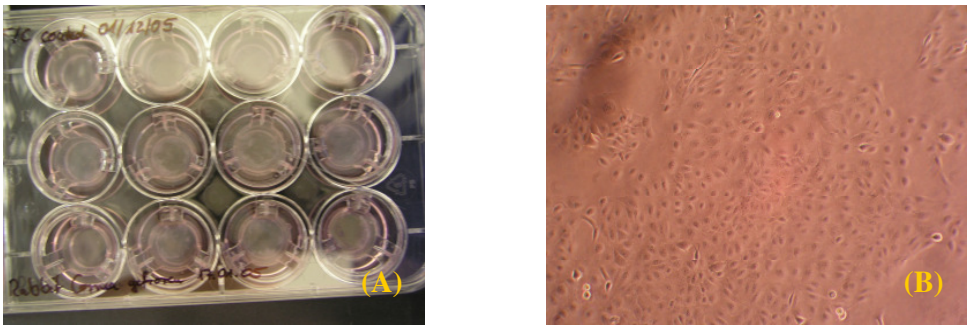


Figure 4: *macroscopic (A) and microscopic (B) images of* in vitro *cell models*

Bioelectrical parameters

To evaluate the barrier properties of *in vitro* models, two parameters are generally monitored: The transepithelial electrical resistance (TEER) and the potential difference (PD). While the TEER value is an indicator for the tightness of the (epithelial) barrier, generated by the cells and their tight junctions, the PD represents the activity of ion-transporters. The TEER is usually expressed in $\Omega*cm^2$, the PD in mV, which allows to standardize and transfer the measured values to various barrier and cell systems. Both values are corrected for a background, contributed by the filter membrane of *in vitro* cell culture systems (TEER value) or the conductance of the medium (PD).

TEER and PD are measured best with an electrical voltohmmeter equipped with "chopstick" electrodes that suit the permeable cell culture inserts (e.g., Corning Transwell®, Greiner Thinserts®, NUNC CC Inserts®). A suitable device is provided by World Precision Instruments (EVOM with STX-2 electrodes). The device is shown in figure 5. TEER values are also assessed as standard procedure in the experiments described herein to control the integrity of the cell layers and tissues used.

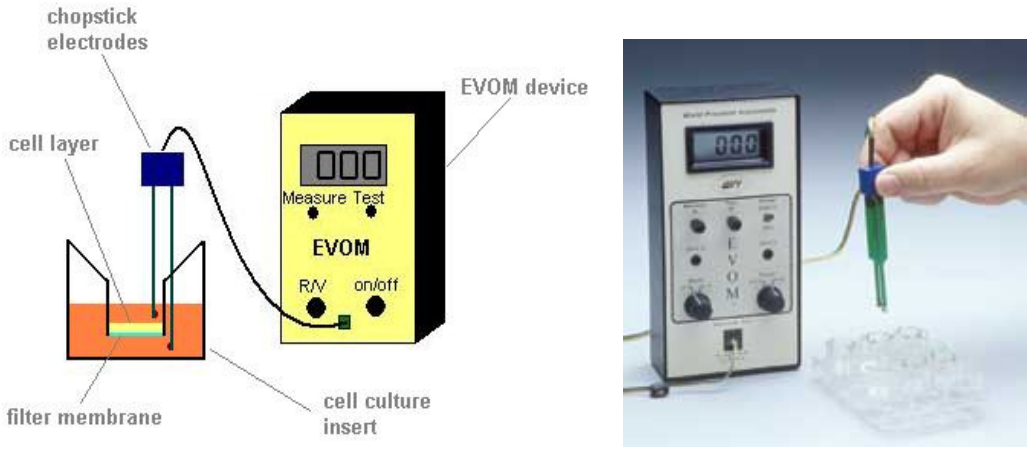


Figure 5: *Scheme of an EVOM device and its application [photo taken from the WPI web page]*

Aim of this work

This work focuses on several aspects of ocular drug delivery research. On the one hand, a new therapeutic compound, moxaverine-hydrochloride, should be examined for its penetration behavior into the eye and its various tissues, on the other hand, a suitable cell culture system should be established to facilitate drug delivery research and reduce the number of animal experiments.

In this work, an attempt was made to establish a primary human corneal epithelial cell model as a tool for drug penetration studies.

Since, as already mentioned, a human *in vitro* system resembles the *in vivo* situation best, the set-up of a human primary corneal epithelial cell model is highly interesting for scientific and biopharmaceutical research.

To evaluate the available cell culture models, a comparison of the different properties of the various cell models is another scope of this work. So far, different cell culture systems for different applications are used side-by-side in research. To find the model most suitable for drug transport studies, we compared the different cell culture models for the first time using identical conditions.

According to this scope, the presented thesis is subdivided in 5 chapters, each dealing with a different aspect of the evaluation of the cell culture system and our new drug compound, moxaverine-hydrochloride:

1. The *in vivo* evaluation of moxaverine-hydrochloride, its ocular distribution and bioavailability are examined in Chapter 1.
2. In Chapter 2, the studies undertaken to establish a human primary corneal cell culture model are summarized.

3. For the comparison and evaluation of different corneal cell models, drug transport studies, employing a portfolio of different marker substances, were performed. Chapter 3 discusses the findings of the study and scores the different systems under investigation.
4. In Chapter 4, the commonly used HCE-T cell model is compared to our *ex vivo* system, generated using excised human cornea. Differences in the molecular-biological layout are pointed out. Main focus of this chapter is on the MDR-transporter spectrum.
5. Further examinations and findings are summarized in Chapter 5.

References

[1] Vander A, Sherman J, Luciano D: human physiology 7th edition, WCB McFraw-Hill, Boston, MA

[2] http://education.vetmed.vt.edu/Curriculum/VM8054/EYE/EYEDEMO.HTM

[3] Klyce S: Electrical profiles in the corneal epithelium, J Physiol 266, 407-429, 1972

[4] Klyce SD: Enhancing fluid secretion by the corneal epithelium, Invest Ophthalmol Vis Sci 16, 968-973, 1977

[5] Mutschler E, Geisslinger G, Kroemer HK, Schäfer-Korting M: Arzneimittelwirkungen; Lehrbuch der Pharmakologie und Toxikologie, 8th edition, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, Germany, 2001

[6] Borderie VM, Kantelip BM, Delbosc BY, Oppermann MT, Laroche L: Morphology, histology, and ultrastructure of human C13 organ-cultured corneas, Cornea 14(3), 300-310, 1995

[7] Nishida T: Chapter I: Cornea in Krachmer JH (Editor), Cornea, St. Louis, Mosby 1997

[8] Radda TM, Menzel EJ: Collagene types in human cornea and sclera, Fortschr Ophthalmol 79(5), 460-462, 1983

[9] Ahmed I, Patton TF: importance of the noncorneal absorption route in topical ophthalmic drug delivery. Invest Ophthalmol Vis Sci: 26, 584-587, 1985

[10] Sasaki H, Ichikawa K, Yamamura K, Nishida K, Nakamura J: Ocular membrane permeability of hydrophilic drugs for ocular peptide delivery, J Pharm Pharmacol 49, 135-139, 1997

[11] Jaervinen T, Jaervinen T, Urtti A: Ocular absorption following topical drug delivery, Adv Drug Deliv Rev 16, 3-19, 1995

[12] Russell WMS, Burch RL: The Principles of Humane Experimental Technique, Charles Thomas Publishers, 1959 (online issue available at: http://altweb.jhsph.edu/publications/humane_exp/het-toc.htm)

[13] Kahn CR, Young E, Lee IH, Rhim JS: Human corneal epithelial primary cultures and cell lines with extended life span: in vitro model for ocular studies. Invest Ophthalmol Vis Sci: 34, 3429-3441, 1993

[14] Neiderkorn JY, Meyer DR, Ubelaker JE, Martin JH: Ultrastructural and immunohistological characterization of the SIRC corneal cell line. In Vitro Cell Dev Biol: 26, 923-930, 1990

[15] Araki-Sasaki K, Ohashi Y, Sasabe T, Hayashi K, Watanabe H, Tano Y, Handa H: An SV40-immortalized human corneal epithelial cell line and characterization. Invest Ophthalmol Vis Sci: 36, 614-621, 1995

[16] Offord EA, Sharif NA, Mace K, Tromvoukis Y, Spillare EA, Avanti O, Howe WE, Pfeifer AM: immortalized human corneal epithelial cells for ocular toxicity and inflammation studies. Invest Ophthalmol Vis Sci: 40, 1091-1101, 1999

[17] Mohan RR, Possin DE, Mohan RR, Sinha S, Wilson SE: Development of genetically engineered tet HPV16-E6/E7 transduced human corneal epithelial clones having tight regulation of proliferation and normal differentiation. Exp Eye Res: 77, 395-407, 2003

[18] Saha P, Kim K, Lee VHL: A primary culture model of rabbit conjunctival epithelial cells exhibiting tight barrier properties. Curr Eye Res: 15, 1163-1169,1996

[19] Chang JE, Basu SK, Lee VH: Air-interface promotes the formation of tight corneal epithelial cell layers for drug transport studies. Pharm Res: 17, 670-676, 2000

[20] Audus KL, Bartel RL, Hidalgo IJ, Borchardt RT: The use of cultured epithelial and endothelial cells for drug transport and metabolism studies. Pharm Res: 7, 435-451, 1990

Chapter 1

Tissue Distribution of Moxaverine-Hydrochloride in the Rabbit Eye and Plasma

Parts of this chapter have been published in:

Ulrich Becker, Carsten Ehrhardt, Ulrich F. Schaefer, Hovhannes J. Gukasyan, Kwang-Jin Kim, Vincent H. L. Lee and Claus-Michael Lehr (2005) Tissue Distribution of Moxaverine-Hydrochloride in the Rabbit Eye and Plasma. *J Ocul Pharmacol Ther* **21**: 210-216

Abstract

Objective: The aim of this study was to determine the tissue distribution and epithelial penetration of moxaverine-hydrochloride (MOX) in the rabbit eye.

Methods: For systemic application, a radioactively labeled MOX solution was injected into the ear vein of Dutch-belted pigmented male rabbits. For topical dosing, an identical solution was administered. At pre-determined time points, rabbits were sacrificed, the eye dissected, and the amount of MOX in the ocular tissues measured. To examine the MOX permeability across the corneal epithelium, transport studies using rabbit corneal epithelial cell culture were conducted and the respective apparent permeability coefficient in absorptive (a to b) or secretive (b to a) direction was calculated.

Results: Topical delivery resulted in high concentrations of MOX in cornea and conjunctiva, while other tissues of the anterior part yielded lower MOX concentrations. In the tissues of the posterior part, high amounts were detected in the retina. Plasma levels were low. The apparent permeability coefficient across corneal epithelial cell layers was in the range of 10^{-5} cm/s, exhibiting no apparent directionality.

Conclusions: A topical dosing of MOX to posterior regions of the eye seems feasible. MOX levels in the posterior part of the eye were remarkably high, without causing stringent plasma levels. The high apparent permeability coefficient of MOX across the corneal epithelial cell layers might be due to the lipophilic nature of the drug and was in the range of other compounds with comparable physicochemical properties.

Introduction

Targeting drugs to the posterior part of the mammalian eye via topical application remains a formidable task and is of major interest for ophthalmic research, because of the easy application for the patient and considerably low systemic availability, which might alleviate unwanted side effects.

In addition, to opt for the ocular route of drug delivery might avoid drug degradation caused by the first pass effect occurring in the liver. Moreover, it is possible to target drugs to several sites or tissues of interest [1].

Potential disadvantages of ocular drug delivery may include the rather low applicable volume and the rapid drainage of the drug solution through the naso-lacrimal duct. The comparison of advantages and disadvantages of topical (ocular) dosing and systemic application is a rewarding field of research, as is the evaluation of the distributional profiles of old and new drug entities.

Moxaverine-hydrochloride (MOX) is a derivative of papaverine-hydrochloride, a component of the chyle of the poppy (*papaver somniferum*) used as muscle relaxant, but its efficacy in the ophthalmic clinic is unknown. Compared to papaverine-hydrochloride, MOX offers enhanced solubility in hydrophilic or aqueous solvents and a lower toxicity [2,3]. The chemical structure of MOX is shown in Figure 1.

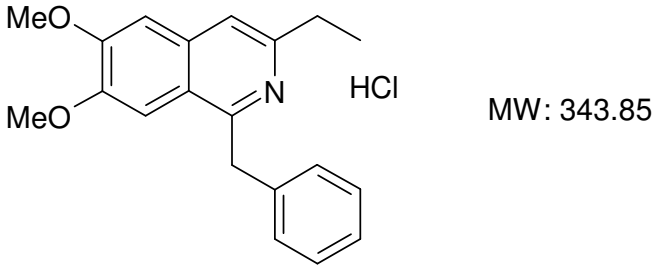


Figure 1: *Chemical structure of moxaverine-hydrochloride*

The synthesis of MOX (1-benzyl-3-ethyl-6, 7-dimethoxy-isoquinoline) was first described in 1962 [4].

Physicochemical properties of interest for MOX include a high lipophilicity (log $P_{pH\ 7.4}$= 4.017), a pK_A-value of 6.75 for the nitrogen-atom and a high amount of protein binding.

In the clinic, the hydrochloride-salt is used instead of the free, lipophilic base, due to its increased solubility. The pharmacodynamic effects of MOX include: increased rheology of erythrocytes; blood vessel relaxation in the brain as well as in the peripheral organs (e.g., arms and legs); and spasmolysis of the vessel musculature [5,6]. The vessel relaxation and spasmolytic properties of MOX seem to be of major interest in ocular application, in addition to the decreased viscosity of the blood, which is useful in therapy of circulatory disorders. Other mechanisms of action include an inhibition of the enzyme phosphodiesterase (PDE) [6 - 11] and coupling with calmodulin resulting in a stronger inhibition of the calmodulin dependant PDE [12, 13].

In recent years, considerable efforts have been made to develop *in vitro* models that allow reliable mechanistic examinations of cellular and molecular processes involved in the absorption of xenobiotics. The gastrointestinal tract has been examined for that purpose very intensively, whereas ocular cell culture models have also been used as a tool to examine drug transport mechanisms. In this context, protocols have been developed for the isolation and cultivation of primary corneal and conjunctival epithelial cells [14, 15] and continuous cell lines have been established [16, 17]. Because cells in primary culture resemble the native tissue closer than cell lines, we also conducted our studies using primary cultured corneal epithelial cells isolated from male, Dutch-belted pigmented rabbits. Culture conditions are known to

be highly relevant for proliferation and differentiation of various epithelial cells. Adapting from the culture techniques for airway epithelial cells [18, 19], air-interface culture (AIC) of ocular epithelial cell models yielding a very tight and well-differentiated cell layer [14, 15], has been developed and utilized in our laboratories.

In this study, we determined whether MOX, a drug of potential interest for ophthalmic treatment/research, could be delivered to tissues of interest by topical application to the eye and also estimated the apparent permeability coefficients across the primary cultured corneal epithelial cell layers.

Materials and methods

Materials

Moxaverine-hydrochloride (MOX) was a gift from Ursapharm Arzneimittel GmbH & Co. KG (Saarbrücken, Germany). ^{14}[C]-Radiolabeled MOX (specific activity 30 mCi/mmol) was synthesized by BIOTREND (Cologne, Germany). All other materials were of the highest grade available commercially. Eutha-6 CII sodium pentobarbital solution was purchased from Western Medical Supply (Arcadia, CA). Triton X-100, anhydrous citric acid, protease type XIV and DNase I were from Sigma (St. Louis, MO). Sodium hydroxide was from Curtin Matheson Scientific (Houston, TX) and PBS (10x) from Gibco (Grand Island, NY). Scintillation cocktail (Econo-Safe) was purchased from Research Products Intl. (Mount Prospect, IL) and heparin 5000 IU/mL from Elkins-Sinn (Cherry Hill, NJ). Transwell filter inserts (catalog number 3460) were from Costar (San Francisco, CA). Male, Dutch-belted pigmented rabbits, weighing ~ 2.2 kg, were purchased from Irish Farms (Los Angeles, CA). The animals were utilized according the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Methods

In vivo administration:

For the *in vivo* studies, 10 mL of a citrate buffer (44.06 mM citric acid, 80.6 mM NaOH, pH = 3; 280 mOsm) were spiked with 11 µL MOX-radiotracer. The total concentration of MOX in the eye drop preparation was 1.28×10^{-3} M. The concentration was chosen to make the achievement of significant concentrations in the aqueous humor most likely. The iso-osmotic pressure mainly limited the

concentration of MOX in the eye drop preparation. However, 10^{-7} M has been found to be a minimal active concentration by Berndt et al. [8] using a porcine ateria femoralis preparation. A modification of the protocol of Schoenwald and Steward [20] was used for ocular administration. Briefly, the rabbits were placed in restrainers and 50 µL of the isotonic drug solution, spiked with a radioactive tracer as described above, was topically applied to each eye. For the determination of the drugs bioavailability after intravenous administration, an identical solution was injected to the marginal ear vein of the animal. At predetermined time points up to 120 min, blood samples were taken from the artery in the central region of the rabbit ear. Blood coagulation was prevented with heparin and the blood cells were separated from plasma by centrifugation. Subsequently, rabbits were sacrificed by an overdose of Eutha-6 CII given intravenously. From one eye, samples of aqueous humor and vitreous body were taken; the other eye was excised as a whole from which various ocular tissues (e.g., cornea, conjunctiva, lens, iris/ciliary body, retina, sclera) were dissected. For the isolation of retina and sclera, the vitreous body was removed from the eye using a pair of forceps. Subsequently, the outer tissues were flattened by eight incisions along the sclera-iris-rim. The retina, containing the choroids, was scraped off using a scalpel blade.

Individual tissues were then lysed with 1 mL solubilizer mixture (comprised of Triton X-100 (15% v/v) and 0.5 N NaOH) for 12 hours. Scintillation cocktail was added to the vials containing the individual tissue lysates and the radioactivity of each lysed tissue sample was determined in a scintillation counter (LS 1801, Beckman, Fullerton, CA).

In vitro permeability studies:

A primary culture of rabbit corneal epithelial cell layers (rbCrECL) was used. This *in vitro* model has been described previously by Chang *et al.* [14] and has been slightly adapted for our purposes. Briefly, pigmented male Dutch-belted rabbits were sacrificed by an intravenous overdose of Eutha-6 CII solution (120 mg pentobarbital-sodium/kg body weight) via the marginal vein of the left rabbit ear. The entire eye was carefully removed by an aseptic method. The corneal epithelial cell layers were exposed to a 0.2% solution of protease type XIV for 45 min at 37°C before being gently scraped off. Protease activity was stopped by adding supplemented minimum essential medium (S-MEM) containing 10% fetal bovine serum (FBS). The addition of excess proteins caused a quenching of enzymatic activity. The following removal of the medium completely disposed of the protease.

Cell aggregation was reduced by including 0.75 mg/mL DNase I in the same solution. Isolated cells were washed and purified further by passing them through a 40-µm cell strainer. The resulting corneal epithelial cells (viability >95%, tested by trypan blue exclusion assay) were then plated onto 1.13 cm^2 polyester Transwell filter inserts (12 mm in diameter, pore size 0.4 µm), precoated with a mixture of rat-tail collagen (type I, 1.8 µg/cm^2) and human fibronectin (0.6 µg/cm^2). DMEM/F12, supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), gentamicin (50 µg/mL), insulin-transferrin-selenium premix (10 µL/mL ITS^+, BD Biosciences, San Jose, CA), bovine pituitary extract (30 µg/mL), epidermal growth factor (EGF, 1 ng/mL) and hydrocortisone (0.36 µg/mL), was used as culture medium. Cells were maintained in a humidified incubator at 37°C in 5% CO_2 atmosphere. Air-interfaced culture (AIC) conditions were created by removing media from the apical compartment and keeping 700 µL of media in the basolateral compartment starting

one day after cell plating. Culture media were changed on a daily basis after bioelectrical parameters were assessed. The bioelectric parameters of transepithelial electrical resistance (TEER) and potential difference (PD) were measured with an epithelial voltohmmeter (EVOM), equipped with STX-2 chopstick electrodes (World Precision Instruments, Sarasota, FL). TEER and PD were corrected for the background values contributed by the empty filter and culture medium.

Transport studies:

In vitro transport experiments were performed on day 7 to 10 in culture. All solutions were used prewarmed to 37°C. The cell layers were washed with a modified Krebs-Ringer solution with bicarbonate buffer (KRB, 1.5 mM K_2HPO_4, 3 mM KCl, 4 mM glucose, 142 mM NaCl, 10.07 mM 4-(2-Hydroxyethyl)-piperazine-1-ethansulfonic acid (HEPES), 1.4 mM $CaCl_2$, 2.56 mM $MgCl_2$, pH = 7.4). The transport experiments were initiated by exchanging the KRB of the respective donor side with a same volume of isotonic radiolabeled MOX solution (1mCi ^{14}C-MOX / L). The volumes in the apical and basolateral chamber were, respectively, 500 µL and 1500 µL.

Samples (100 µL) were drawn from the corresponding receiver compartment every 30 minutes up to 3 hours. Sampled volumes were replaced by the same amount of fresh pre-warmed KRB solution. Samples were mixed with scintillation cocktail and assayed for their radioactivity using a scintillation counter. To determine the initial concentration, 20-µL samples of the test solution were assayed for radioactivity.

All transport experiments were performed with and without addition of benzalkonium chloride/EDTA, a potential preservative. Concentrations for the

preservatives were chosen according to recommendations given in the German Drug Codex (DAC) [23], that is, 0.01 % (w/v) for benzalkonium chloride and 0.2% (w/v) for EDTA.

Experiments were performed in triplicate. Both TEER and PD values were assessed before and after the drug permeation experiments to assess the integrity of the cell layers.

The apparent permeability was calculated as flux of MOX versus time, using the relation,

$$P_{app} = \frac{J}{c_0 * A} \qquad \textbf{(Eq. 1)}$$

where, J = MOX flux (mol/s), c_0 = initial MOX concentration in the donor solution (mol/cm^3) and A = surface area of the cultured corneal epithelial cell layer (cm^2).

Results

In vivo experiments:

The results of the *in vivo* study of MOX accumulation in the anterior (i.e., conjunctiva, cornea, aqueous humor, iris and ciliary body, lens) and posterior parts (i.e., sclera, retina) of the eye are respectively shown in Figures 2 and 3. Drug amounts found in the plasma (representing systemic bioavailability) are also given in Figure 3.

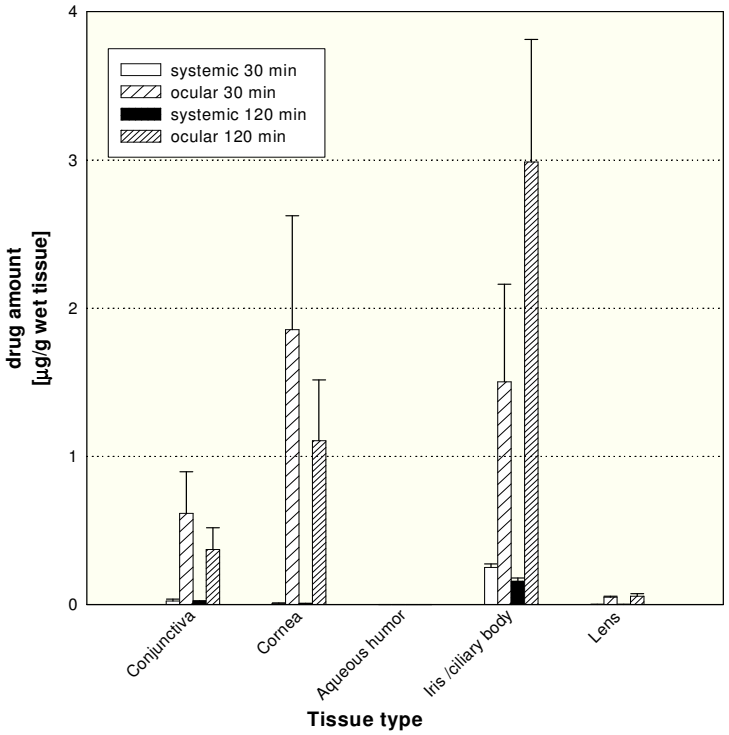


Figure 2: *Tissue distribution of moxaverine-hydrochloride in the anterior parts of the eye following systemic vs. ocular delivery. Experiments were performed on 3 rabbits for each time point, except for systemic application 30 min (n=2).*

The error bars reflect standard deviations.

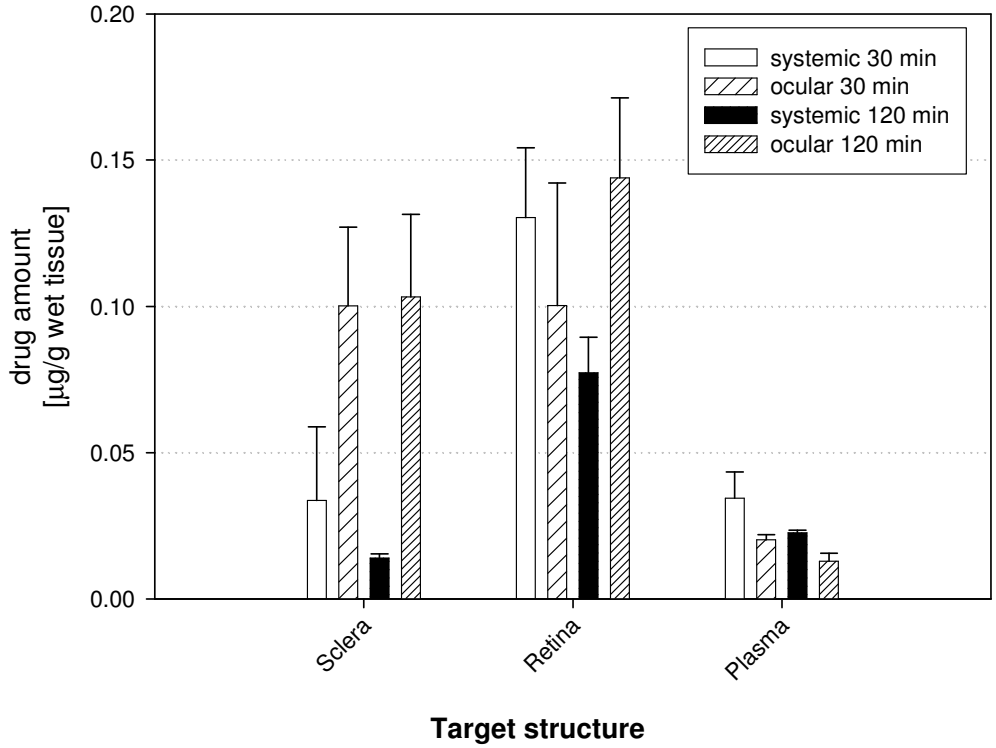


Figure 3: *Tissue distribution of moxaverine-hydrochloride in the posterior parts of the eye and plasma. Experiments were performed on 3 rabbits for each time point, except for systemic application 30 min (n=2).*

The error bars reflect standard deviations

After topical administration, the highest levels of MOX were found in the anterior parts of the eye, i.e., conjunctiva, cornea and iris/ciliary body. The lens and aqueous humor contained no measurable amounts of drug.

For the posterior parts of the eye, the MOX concentration within the retina at 30 min after topical administration was slightly lower than those attained via systemic application. After 2 hours, drug content of the retina decreased after systemic application, whereas drug content increased in the same tissue after ocular dosing. It

should be noted that the plasma values after topical dosing are significantly lower compared to those observed with systemic dosing.

Amounts of MOX in the sclera at both 30 and 120 min in after intravenous application, were lower than those found with ocular administration.

In vitro experiments:

The results of the *in vitro* transport studies conducted with primary rabbit corneal epithelial cell layers are shown in Table 1. The P_{app} values for both absorptive and secretive directions were in the same range (i.e., ~ 1.4x 10^{-5} cm/sec), indicating that no active transport mechanism in the corneal epithelium seems to be involved in the absorption of MOX. The observed P_{app} values were in the same order of magnitude compared to other substances with similar physicochemical properties (Table 2).

Table 1: *P_{app} values of moxaverine-hydrochloride across primary rabbit corneal epithelial cell layers (mean ± SD, n = 9)*

Direction of drug transport	**P_{app} (x10^{-5} cm/sec) + TEER (after experiment)**	
	+ benzalkonium chloride/EDTA	*- benzalkonium chloride/EDTA*
Absorptive (a to b)	1.28 ± 0.41 TEER: 2203 ± 202 Ω * cm^2	1.38 ± 0.41 TEER: 2288 ± 358 Ω * cm^2
Secretive (b to a)	1.33 ± 0.22 TEER: 2137 ± 311 Ω * cm^2	1.43 ± 0.94 TEER: 1939 ± 640 Ω * cm^2

Table 2: Comparison of P_{app} values (for details, see [27])

All P_{app} were examined for absorption (i.e., a to b direction) across rabbit cornea.

Substance	**M_W**	**Log P**	**pH**	***P_{app}*(x 10^{-5} cm/sec)**
Corynanthine	354	3.01	7.70	1.10
4, 6-dichloroethoxzolamide	283	3.70	7.65	3.90
Flurbiprofen	244	3.75	7.40	2.10
Progesterone	314	3.78	7.65	2.00
Progesterone	314	3.78	7.50	1.80
Propranolol	259	2.75	7.50	3.10
Penbutolol	291	4.04	7.00	2.20
Moxaverine-HCl	344	4.02	7.40	1.38

The preservative benzalkonium chloride/EDTA does not appear to alter MOX transport across primary cultured rabbit corneal epithelial cell layers. We note, however, that a decrease of ~23% in TEER values of the cell layers during the experiment was observed in the experiments carried out using the preservative containing buffer. This lowering of TEER may have occurred because of complexation of Ca^{2+} with EDTA, subsequently loosening tight junctions. It appears that such decrease in TEER does not result in an increased P_{app} of MOX, which is already high at baseline.

Discussion

This study provides first evidence of the feasibility of a targeted delivery of moxaverine-hydrochloride to the posterior parts of the eye after topical instillation. Except for the cornea and conjunctiva, which were in close and direct contact with the applied drug solution, only low drug levels could be found in the anterior part, because of either low affinity to the tissue or the high turnover rate of 0.21 to 0.34 ml/min/mm Hg for the aqueous humor [24]. This is indicative for low local side effects during topical dosing in clinical treatment of diseases of the posterior part of the eye.

For the posterior part, comparably high amounts of MOX could be detected. Particularly for the compartment of interest, the retina, detectable drug amounts are a promising observation. Ocular dosing shows a fast accumulation of the drug, even increasing values throughout the experimental period. By contrast, systemic dosing leads to a decrease in the drug level during the same time period, resulting in part to high clearance rates of MOX from the systemic circulation [25]. For the human body, a high rate of metabolism was found (iphar 87/253 study). MOX is degraded to an aldehyde and an alcohol structure. The pharmacologic activity of these metabolites is unknown. The presence of metabolites in our study is most likely, but was not examined.

For drug penetration, the following ways of distribution have been reported [26]: The direct corneal pathway, the conjunctival-scleral pathway, distribution by the local vasculature and the re-entry from general circulation after drug absorption. The contribution of the general circulation to the drugs penetration to posterior compartments in this case is very low, reflected by the low concentration values in the

plasma. The effect of the local vasculature has not been examined. Very likely is the diffusion by the conjunctival-scleral pathway, because it has been shown to be a way of diffusion for substances with high molecular weight. We have also shown that cornea is not a permeability barrier for the absorption of MOX into the eye. It is most likely that the drug is absorbed in this direct way.

The significantly lower drug levels in plasma are worthy of particular note. Further studies are required to determine whether the detected drug levels can achieve the desired pharmacodynamic effect.

The permeability data of MOX across the primary corneal epithelial cell model did not reveal any directionality, indicating that MOX has no affinity to transport proteins such as P-glycoprotein. Moreover, the P_{app} for MOX is in the range of 10^{-5} cm/sec. Thus, the corneal epithelium may not be a critical barrier for the delivery of MOX. Addition of benzalkonium chloride/EDTA may have led to loosening of tight junctions of the cell layer, indicated by a decrease of the TEER values by the inclusion of benzalkonium. Despite the observed decrease in TEER, P_{app} of MOX across the cell layer was not affected significantly. Thus, we may conclude that absorption of MOX across the corneal epithelial barrier occurs mainly via transcellular passive diffusion. An enhancing effect of preservatives on absorption of hydrophilic drugs due to complexation of Ca^{2+} with EDTA by opening of tight junctions has been reported earlier [21,22].

In conclusion, we showed herein that a potentially new drug in ophthalmology, moxaverine-hydrochloride, reaches significant drug concentrations in posterior compartments of the eye after topical application and that absorption of this compound is not mediated by active transport processes.

Acknowledgements

This work was supported by Ursapharm Arzneimittel GmbH & Co. KG, Saarbrücken, Germany and by National Institutes of Health grants EY12356 (VHLL), HL64365 (KJK and VHLL) and HL38658 (KJK). Ms. Dorothea Groß and Dr. Klaus Eschmann at Ursapharm are thanked for their helpful advice and valuable discussions.

References

[1] Newsome A, Stern R. Pilocarpine adsorption by serum and ocular tissues. *Am. J. Ophthalmol.*; 77: 918-922, 1974

[2] IBR-Report 1-4-190-87: Acute oral toxicity of papaverine-hydrochloride in rats, Ursapharm, 1987

[3] IBR-Report 1-4-191-87: Acute oral toxicity of moxaverine-hydrochloride in rats, Ursapharm, 1987

[4] Chemical Abstracts, 1965, 11522a, Patent Orgamol

[5] Bayer R, Plewa S, Borescu E, Claus W. Filterablility of human erythrocytes. Drug induced prevention of aging in vitro. Arzneim. Forsch.; 38: 1765-1769, 1988

[6] Berndt SF, Schulz HU. Effects of papaverine derivatives in cyclic-3', 5'-AMP phosphodiesterase and relaxation of rabbit ileum. J. Neural. Transm. Suppl.; 11: 187-193, 1972

[7] Berg G, Andersson RGG, Ryden G. Effects of different phoshodiesterase-inhibiting drugs on human pregnant myometrium: an in vivo study. Arch. In. Pharmacodyn. Ther.; 290: 288-292, 1987

[8] Berndt SF, Schulz HU, Stock, K. Influence of papaverine derivatives on phosphodiesterase activity, cAMP-levels and relaxing effect on rabbit ileum. Naunyn-Schmiedberg's Arch. Exp. Pathol. Pharm.; 294: 271-275, 1976

[9] Kukovetz WR, Poech G. Inhibition of cAMP-phosphodiesterase as a possible mode of action of papaverine and similarly acting drugs. Naunyn-Schmiedberg's Arch. Pharmacol.; 267: 189-194, 1970

[10] Lugnier C, Stoclet JC. Inhibition by papaverine of cGMP and cAMP phosphodiesterase from rat heart. Biochem. Pharmacol.; 23:3071-3074, 1974

[11] Poech G, Umfahrer W. Differentiation of intestinal smooth muscle relaxation caused by drugs that inhibit phosphodiesterase. Naunyn-Schmiedberg's Arch. Pharmacol.; 293: 257-268, 1976

[12] Mannhold R. Inhibition of calmodulin dependant cyclic-3', 5'-AMP-phosphordiesterase by moxaverine and papaverine. Arzneim.-Forsch.; 38: 1806-1808, 1988

[13] Mannhold R. Calmodulin antagonists: chemistry, molecular pharmacology and therapeutic applications. Drugs Today; 28: 175-199, 1992

[14] Chang J, Basu S, Lee, VHL. Air-interface condition promotes the formation of tight corneal epithelial cell layers for drug transport studies. Pharm. Res.; 17: 670-676, 2000

[15] Yang JJ, Ueda H, Kim KJ, Lee VHL. Meeting future challenges in topical ocular drug delivery: development of an air-interfaced primary culture of rabbit conjunctival epithelial cells on a permeable support for drug transport studies. J. Control. Rel.; 65: 1-11, 2000

[16] Araki-Sasaki K, Ohashi Y, Sasabe T, Hayashi K, Watanabe H, Tano Y, Handa H. An SV40-immortalized human corneal epithelial cell line and its characterization. Invest. Ophthalmol. Vis. Sci., 36: 614-621, 1995

[17] Goskonda VR, Khan MA, Hutak CM, Reddy IK. Permeability characteristics of novel mydriatic agents using an in vitro cell culture model that utilizes SIRC rabbit corneal cells. J. Pharm. Sci., 88: 180-184, 1999

[18] Yamaya M, Finkbeiner WE, Chu SY, Widdicombe JH. Differentiated structure and function of cultures from human tracheal epithelium. Am J. Physiol.; 262: L713-L724, 1992

[19] Mathias NR, Kim KJ, Robinson TW, Lee VHL. Development and characterization of rabbit tracheal epithelial cell monolayer models for drug transport studies. Pharm. Res.; 12: 1499-1505, 1995

[20] Schoenwald RD, Steward P. Effect of particle size on ophthalmic bioavailability of dexamethasone suspensions in rabbits. J. Pharm. Sci.; 69: 391-394, 1980

[21] Sasaki H, Nagano T, Yamamura K, Nishida K, Nakamura J. Ophthalmic preservatives as absorption promoters for ocular drug delivery. J. Pharm. Pharmacol.; 47: 703-707, 1995

[22] Lopez-Bernal D, Ubels L. Quantitative evaluation of the corneal epithelial barrier: effect of artificial tears and preservatives. Curr. Eye Res.; 10: 645-656, 1991

[23] Kommission Deutscher Arzneimittel-Codex. Angaben zur Konservierung von Augentropfen. ABDA-Bundesvereinigung Deutscher Apothekerverbaende. Deutscher Arzneimittel-Codex Band I, Eschborn; Govi-Verlag Pharmazeutischer Verlag GmbH: 1-2, 2000

[24] Pfeiffer RL, Pohm-Thorsen, Corcoran K. Models in ophthalmology and vision research. Manning PJ, Ringler DH, Newcomer CE. The biology of the laboratory rabbit second edition. Burlington, MA; Academic Press;: 414-415, 1994

[25] Kammerer E, Wegener T, Schmidt GP. Pharmakologie und Klinik von Moxaverin. Jatros Kardiologie, 5/6: 3-10, 1994

(26) Ahmed I, Patton TF. Importance of the noncorneal absorption route in topical ophthalmic drug delivery. Invest Ophthalmol Vis Sci., 26: 584-587, 1985

[27] Prausnitz MR, Noonan JS. Permeability of cornea, sclera and conjunctiva: a literature analysis for drug delivery to the eye. J Pharm. Sci.; 87: 1479-1488, 1998

Chapter 2

Set-up of a primary human corneal epithelial cell culture system

Introduction

Because animal testing is evaluated controversionally, other more accepted and easier methods have been established since cell culture started in the early 20th century [1, 2].

Major drawbacks for animal testing are the high maintenance costs and efforts. Scientifically, the complexity of the "test" system and the high variation of the outcomes are major points for criticism. Last, but not least does animal testing have a bad reputation and low acceptance in the modern Western society. Therefore, lots of efforts have been put into the development of cell culture systems that replace laboratory animals in scientific studies.

For ocular tissues, two major aspects are of importance: ocular irritation and ocular drug penetration and delivery.

A major tool for ocular irritation testing is the Draize test, developed in the 1940s by Draize *et al.* [3]. Briefly, in this test, laboratory rabbits are dosed ocularly with the test substance and changes on the dosed eye are observed and compared with the untreated rabbit eye over a predetermined time.

For ocular drug delivery, *in vivo* studies on various animals have been a standard tool and are still performed today [4, 5, 6, 7].

To get an easier to handle tool, various *ex vivo* and *in vitro* cell models have been developed. Main advantages of these models are

(1) Fixed conditions for the examination of cell growth, differentiation, and response to (chemical) agents
(2) Easy access to the apical (i.e., front part) and basolateral (i.e., rear part) side of the cells or tissue
(3) Absence of foreign cells from unwanted tissue
(4) Quick and easy determination of data on drug transport processes on ocular irritation or damage
(5) Reduction of animal experiments

A variety of corneal epithelial cell cultures have been established in recent years. Besides the immortalized cell culture models that have an extended life span, improved cell growth characteristics and are easy to handle [8], various attempts have been made to establish primary corneal epithelial cell cultures that better resemble the *in vivo* situation and have other than immortalized cell models not lost original cell properties [9].

Different techniques have been applied to generate these cell models: explantation techniques [10, 11], enzymatic treatments [12, 13] and mechanical processing [14, 15]. Successful primary cultures of the corneal epithelium have been generated for following species: rabbit [10, 12], cow [16], guinea pig [17], rat [18], mouse [31] and pig [30].

In the explantation technique, corneal epithelial cells are gained by outgrowth from tissue pieces, which are placed epithelial side down in a tissue culture treated petri dish. By culturing these pieces in an appropriate medium in an incubator, epithelial cells will grow from the tissue onto the cell culture dish. Ebato *et al.* [11] found that the limbal cells show a better proliferation than cells from the central cornea since the limbus between cornea and sclera is the origin of corneal stem cells [19, 20]. The overgrowth of the epithelial cell layers by fibroblasts from the stroma is considered as a major drawback of this technique. Schneider has developed a method to avoid this overgrowth [21].

For the enzymatic method, a gamut of different proteolytic enzymes [12, 13, 22] has been used to dissociate the cells. Basis of this method is the lysis of the intercellular proteins and to loosen the cell connections, so that single cells of a specific type are yielded.

The mechanical processing uses sheer forces and mincing steps to separate corneal cells. A variety of scissors, scalpels and microtomes have been used for this purpose [14, 23].

Even tough several attempts have been undertaken; so far a successful set up of a human corneal epithelial cell model was not achieved.

In this study, we tried to set up such a model by modifying the techniques described so far and merging these with new findings made in recent years.

Materials and Methods

Materials

Collagenase type II, pronase, protease type XIV, trypsin, ethanol and DNase I were purchased from Sigma (Steinheim, Germany). Dispase II was from Roche Applied Sciences (Mannheim, Germany), MACS MicroBeads (coated with monoclonal mouse anti-human epithelial antigen antibody HEA-125/CD326)were from Miltenyi Biotec (Bergisch Gladbach, Germany).

Scalpel blades (Bayha, model No. 1), surgical scissors and forceps were purchased from Stoss Medica (Saarbrücken, Germany).

Rat tail collagen type I was from Cohesion (Palo Alto, CA, USA) and fibronectin was from BD Bioscience (Heidelberg, Germany). McIlwain tissue chopper was from The Mickle Laboratory Engineering Co. Ltd. (Gomshall, Surrey, Great Britain), Ultra Turrax rotor-stator homogenizer was from IKA (Staufen, Germany).

Corneal rings were left-overs from successful transplantations and a generous gift from the Lions Corneabank, Homburg, Germany. Minimal Essential Medium (MEM) and Dulbecco's Modified Eagle's Medium (DMEM) were from Invitrogen (Niederaula, Germany).

Pattex Blitz Matic cyanoacrylate glue was from Henkel, Düsseldorf, Germany.

All other chemicals were of highest grade available commercially.

Methods

Protease/DNase technique

A technique previously described by Chang *et al.* [12] has been slightly adapted for our purposes. Briefly, the corneal rings exposed to a 0.2% solution of protease type XIV for 45 min at 37°C before gently scraping them off. Protease activity was stopped by adding supplemented minimum essential medium (S-MEM) containing 10% fetal bovine serum (FBS). The addition of excess proteins caused a quenching of enzymatic activity. The following removal of the medium completely disposed of the protease.

Cell aggregation was reduced by including 0.75 mg/mL DNase I in the same solution. Isolated cells were washed and purified further by passing them through a 40-µm cell strainer. The resulting corneal epithelial cells were seeded into 25 cm^2 cell culture flasks precoated with a mixture of rat tail (type I) collagen (1.8 mg/cm^2) and human fibronectin (0.6 mg/cm^2). DMEM/F12, supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), gentamicin (50 µg/mL), insulin-transferrin-selenium premix (10 µg/mL ITS^+, BD Biosciences, San Jose, CA), bovine pituitary extract (30 µg/mL), epidermal growth factor (EGF, 1 ng/mL) and hydrocortisone (0.36 µg/mL), was used as culture medium. Cells were maintained in a humidified incubator at 37°C in 5% CO_2 atmosphere. Culture medium was changed every other day. Cell yield and growth were monitored microscopically

Other enzymatic techniques

All dissociation techniques were tested at 37°C and 4°C for 45 min.

For enzymatic treatment of tissues the following enzymes have been described in literature: collagenase II [29], dispase II [30], pronase [22], and trypsin [22].

Tested concentrations were 1.2 U/mL dispase II, 167 U/mL collagenase II, 167 U/mL collagenase II with 9563 U/mL trypsin, 334 U/mL collagenase II with 19130 U/mL trypsin, in balanced salt solution (BSS; 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 10 mM HEPES, 5.5 mM glucose, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, pH = 7.4), respectively.

For cell isolation, the corneal rings were treated with the respective enzyme for 45 min either at 37°C or 4°C. After incubation corneal rings were attached to the outer bottom surface of glass centrifuge tubes with cyanoacrylate glue. Epithelial cells were then removed from the corneal surface by gentle scraping with a scalpel blade. Harvested cells were washed and purified by passing the solution through a 40-µm cell strainer. For pronase, collagenase II and collagenase II/trypsin, cell clotting was avoided by the use of 0.75 mg/mL DNase I in the first

washing step. Cells were seeded into 25 cm^2 cell culture flasks and maintained with DMEM/F12, supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), gentamicin (50 µg/mL), ITS^+ (10 µL/mL), bovine pituitary extract (30 µg/mL), EGF (1 ng/mL) and hydrocortisone (0.36 µg/mL). Cell growth was monitored microscopically.

For dispase II, the most commonly used enzyme for human tissue in literature, incubation times were altered from 45 min to 12 h, 16 h, and 24 h to increase cell yield.

With dispase II also an alternative method was developed: after treatment of the corneal rings with the enzyme for 16 h at 4°C, corneal rings were washed twice with cold PBS and epithelial cell sheets were further loosened by a 1 min treatment with ethanol 20% at 37°C and subsequent scraping in PBS with a scalpel blade. Scraping and washing liquids were collected and centrifuged for 7 minutes at 1200 rpm (= *g*). Cells were washed with MEM with 10% FCS and then seeded in cell culture flasks with supplemented DMEM/F12 (1:1) mix.

For collagenase/trypsin different enzyme concentrations were tested to optimize the cell yield.

Explantation technique

The explanation technique was performed as previously described [11]. Briefly, sclera was removed from the corneal rings and 9 mm^2 cornea pieces were cultured in fibronectin /collagen-coated Petri dishes with the epithelial side down. As cell culture medium served 3.5 mL of DMEM/F12, supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), gentamicin (50 µg/mL), ITS^+(10 µL/mL), bovine pituitary extract (30 µg/mL), EGF (1 ng/mL) and hydrocortisone (0.36 µg/mL).

After 3 weeks, the sprouted corneal epithelial cells were removed from the Petri dish by gentle scraping with forceps and scalpel blades. The harvested cell sheets were subcultured in collagen/fibronectin coated chamber slides to form confluent cell layers.

Mechanical methods

Two mechanical isolation protocols were developed and combined with enzymatic treatment to increase cell yield:

For the first method, the tissue was cut on a McIlwain tissue chopper and then the 5 µm slices were treated with trypsin/collagenase solution for 1 h at 37°C. Single cells were gained by washing and passing the cell/tissue pellet through a cell strainer. Cells were seeded and monitored as previously described.

In a second protocol, tissues were grinded with an Ultra Turrax rotor-stator homogenizer after removal of scleral tissue, followed by either a 2 h treatment with dispase II at 37°C or an 18 h dispase II treatment at 4°C. Cells were then washed, passed through a cell strainer and seeded as already described.

MACS MicroBead technique

The target of the MicroBead technique was to improve the cell yield by means of a positive selection of epithelial cells. The theoretical procedure is shown in figure 1 and the experimental set-up for cell sorting in figure 2.

Briefly, the tissue was cut on the McIlwain tissue chopper and treated enzymatically with a collagenase II/trypsin mixture at 37°C for 1 h or grinded with the UltraTurrax homogenizer and then treated with dispase II at 4°C for 18 h or at 37°C for 2 h, respectively. The cell suspension was washed with a solution of DNase I (0.75 mg/mL) in MEM with 10% FCS and then with MEM with 10% FCS alone. The suspension was passed through a 40-µm cell strainer. Cells were centrifuged and resuspended in 1.4 mL MEM with 10% FCS and 100 µL MACS MicroBead solution was added. The mixture was kept on an orbital shaker for 30 minutes at 8 – 11°C. The magnetic beads are coated with specific epithelial antibodies and bind to the human epithelial cells. Positive selection of epithelial cells on the supplied

magnetic column followed according to the manufacturer's instructions. Selection was achieved by reconstitution of the column with 3 mL washing buffer (PBS supplemented with 0.5% bovine serum albumin and 146 mM EDTA), followed by passing the cell/MicroBead solution through the column attached to its magnetic holder. The column was washed four times with BSS and the positively selected cells were removed from the detached column with 5 mL S-MEM with 10% FBS. The purification process was repeated with a second, unused magnetic column. Positively selected cells were washed from the column with 2 mL of supplemented DMEM/F12 medium per corneal ring used.

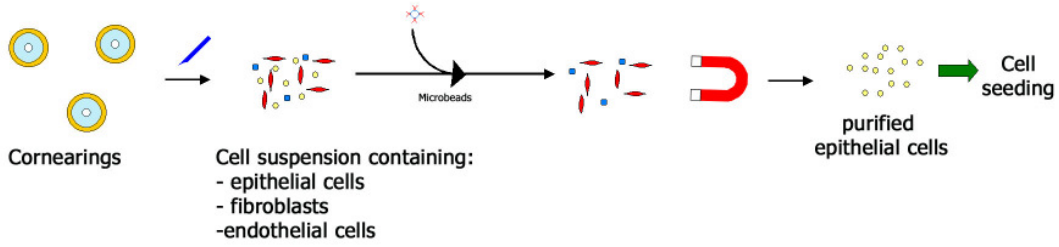


Figure 1: *Isolation steps for the positive selection of corneal epithelial cells.*

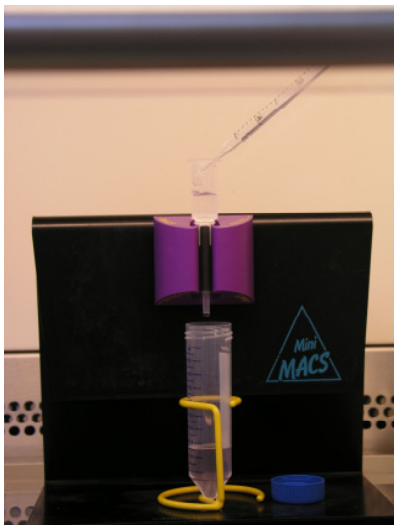


Figure 2: *MACS MicroBead column and magnetic holder*

Results

For all applied techniques, the outcome of experiments was very unsatisfactory. While the protease/DNase and the pronase treatment showed no effect on the corneal cell layers, results could be improved with an appropriate collagenase II/trypsin or dispase II treatment. The evaluation of the enzymatic treatments is summarized in table 1.

Table 1: *Summary of the results of the enzymatic treatment of human cornea*

Enzyme	Result	Comment
Protease	✘	Used with rabbit cornea, but not suitable for human cornea (no cell yield)
Collagenase	(✓)	Only little amount of cells is gained
Trypsin/ Collagenase	✓	187.5 µL/62.5 µL: too much cell damage 93.75 µL/31.25 µL: good results, but small cell amount
Dispase	(✓)	Advantage: no cell clotting, very gentle BUT: small cell amount, alteration of incubation time up to 24 h did not increase efficacy
Pronase	✘	Cell gain unsatisfactory

Figure 3 shows a representative microscopical image after enzymatic isolation of the epithelial cells. Cell number was in general very low. Seeding density was insufficient for the 25 cm^2 cell culture flasks as well as the smaller chamber slides.

Attempts to further increase the cell yield by specific selection of epithelial cell species with magnetic beads did not alter the cell amount in the desired fashion.

Explant techniques showed a higher success and yielded intact cell sheets. However, subcultivated cell sheets lacked sufficient proliferation activity and were unable to grow to confluent cell layers

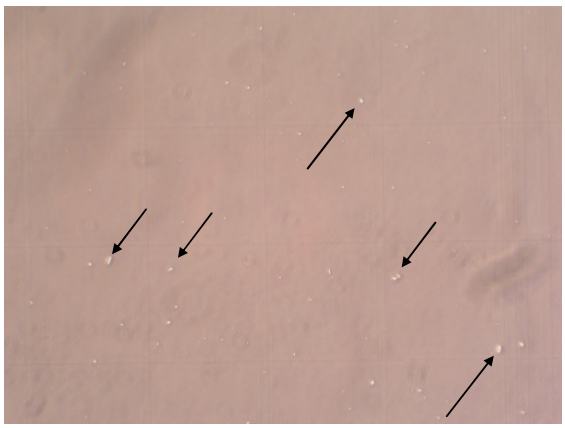


Figure 3: *Light micrograph of isolated cells from corneal epithelial cells*

Discussion

Even though enormous developments have been made in our understanding of cell culture and isolation procedures in recent years, we still encountered major problems while isolating cells from corneal tissue.

Some of these problems are surely to be found in the physiology of the corneal tissue. In addition, the storage time of about 21 days, might contribute to the low activity of the epithelium. Albeit the transplantation efficacy stays unaffected, the corneal epithelium might be critically suppressed in its viability.

As already mentioned in the introduction, the corneal epithelium consists of 9 layers that show different states of differentiation and keratinozation, with only the lowest, first layer consisting of basal cells with mitotic activity. This results in only $1/9^{th}$ of the harvested cells having proliferating activity. So the number of useful cells is *per se* low.

Secondly, the number of cell layers present in the corneal epithelium is highly variable and alters from specimen to specimen. We were able to show the high variance and a possible reduction of cell layers during storage by light micrographs using a standard hematoxyline staining (Figure 4).

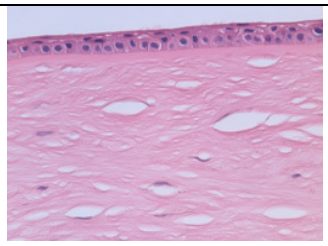
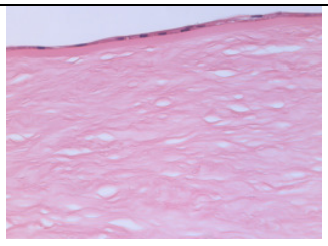
Normal human corneal epithelium	Human corneal epithelium stored for 21 days
	

Figure 4: *Different thickness and reduction of cell layer number in human corneal rings (magnification 400x)*

Many of the applied enzymes have been used with corneal tissue of different species. The spectrum ranges from amphibian (frog) to rodents (rabbits, rats) and mammals (pigs). Since inter-species differences are natural we can expect a diverse composition of the intercellular matrices, which results in different resistance levels to proteolytic enzymes. So it is not surprising to find that protease XIV works well with rabbit cornea [12] but does not affect human tissue.

Even though the magnetic selection of epithelial cells proved very helpful in the isolation of other primary epithelial cells [24], this method did not improve the outcome in our experiments. Since a non-successful binding of the antibody-labeled beads to the epithelial cells can be ruled out, the low cell yield may be based on the following issues:

The amount of available tissue is very low. The number of corneal rings per isolation is generally limited to about 4 tissues. The tissues consist of a corneal ring of about 5 mm diameter, which reduces the number of available cells quite significantly.

The isolation procedure consists of a respectable amount of washing and purification steps that bear the risk of high cell losses. These losses are likely to have occurred in our protocol despite careful preparation.

For the explantation techniques, it is known that corneal cells have a limited capacity of proliferation. The number of possible passages in culture is limited to 2 to 7, depending on the origin of the cells [13]. As separated epithelial sheets do not adhere very well to artificial cell culture surfaces, further obstacles have to be considered and offer a possible explanation for the unsuccessful cultivation activities.

Another point is the selection of cell culture medium. A variety of cell culture media and supplements have been described in literature. Most of these media were tested on cell systems of animal origin. Many media contained fetal bovine serum as supplement [21, 25, 26, 27], which is a standard additive and generally provides all nutrients and hormones

needed. A major drawback in this context is that serum is not present in, at or around corneal tissue under physiological conditions.

Serum-free media was firstly described by Hackworth *et al.* [28] for the culture of porcine corneal epithelium and rabbit corneal epithelium. Chang *et al.* used a refined and very useful serum-free medium on primary rabbit corneal epithelial cell layers [12].

Since this medium proved very successful on these cells and did not contain unphysiological serum, it was chosen for our research. Unfortunately, due to our low cell yields, we were not able to control and optimize the composition of the chosen medium.

References

[1] Harrison RG: Observations on the living developing nerve fiber. Proc Soc Exp Biol Med: 4, 140-143, 1907

[2] Carrel A: On the permanent life of tissues outside the organism. J Exp Med: 15, 516-528, 1912

[3] Draize JH, Woodward G, Calvery HO: Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. Pharmacol. Exp. Ther: 82, 377-390, 1944

[4] Acheampong AA, Shackleton M, John B, Burke J, Wheeler L, Tang-Liu D: Distribution of brimonidine into anterior and posterior tissues of monkey, rabbit and rat eyes. Drug Metab Dispos: 30, 421-429, 2002

[5] Schoenwald RD, Steward P: Effect of particle size on ophthalmic bioavailability of dexamethasone suspensions in rabbits. J Pharm Sci: 69, 391-394, 1980

[6] Lehr CM, Lee YH, Lee VHL: Improved ocular penetration of gentamicin by mucoadhesive polymer carbophil in the pigmented rabbit. Invest Ophthalmol Vis Sci: 35, 2809-2814, 1994

[7] Kiland JA, Croft MA, Gabelt BT, Kaufman P: Atropine reduces but does not eliminate the age-related decline in perfusion outflow facility in monkeys. Exp Eye Res: 64, 831-835, 1997

[8] Daniele N, Halse R, Grinyo E, Yeaman SJ, Shepherd PR: Conditionally immortalized cell lines as model systems for high-throughput biology in drug discovery. Biochem Soc Trans: 30, 800-802, 2002

[9] Tian J, Ishibashi K, Handa JT: The expression of native and cultured RPE grown on different matrices. Physiol Genomics: 17, 170-182, 2004

[10] Stocker FW, Eiring A, Georgiade R, Georgiade N: A tissue culture technique for growing corneal epithelial, stromal and endothelial tissues separately. Am J Ophthalmol: 46, 294-298, 1958

[11] Ebato B, Friend J, Thoft RA: Comparison of central and peripheral human corneal epithelium in tissue culture. Invest Ophthalmol Vis Sci: 28, 1450-1456, 1987

[12] Chang JE, Basu SK, Lee VHL: Air-interface condition promotes the formation of tight corneal epithelial cell layers for drug transport studies. Pharm Res: 17, 670-676, 2000

[13] Germain L, Auger FA, Grandbois E, Guignard R, Giasson M, Boisjoly H, Guerin SL: Reconstructed human cornea produced in vitro by tissue engineering. Pathobiology: 67, 140-147, 1999

[14] Minami Y, Sugihara H, Oono S: Recunstruction of cornea in three-dimensional collagen matrix culture. Invest Ophthalmol Vis Sci: 34, 2316-2324, 1993

[15] Chialbi RGE: Primär- und Subkultur humaner zentraler Corneaepithelzellen. Dissertation, Rheinisch-Westfälische Technische Hochschule Aachen, 1996

[16] Sarkar P, Basu PK, Carré F: Bovine corneal epithelium in tissue culture. Chromosomal stability in aserially subcultured line. Am J Ophthalmol: 61, 553-557, 1966

[17] Favata BV: Growth and behavior of guinea pig iris, retina and cornea in vitro. Am J Ophthalmol: 58, 651-663, 1964

[18] Ellingson DJ, Yao KT: Separation and in vitro growth of mammalian corneal epithelial and endothelial cells. Exp Cell Res: 66, 478-482, 1971

[19] Schermer A, Galvin S, Sun TT: Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol: 103, 49-62, 1986

[20] Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, De Luca M: Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J Cell Biol: 145, 769-782, 1999

[21] Schneider A: Entwicklungen zu einem *In-vitro*-Cornea-Modell im Hinblick auf eine Alternative zum Draize Test, Dissertation, Fraunhofer IRB Verlag, Stuttgart, 1997

[22] Yannariello-Brown J, Hallberg CK, Häberle H, Brysk MM, Jiang Z, Patel JA, Ernst PB, Trocme SD: Cytokine modulation of human corneal epithelial cell ICAM-1 (CD54) expression. Exp Eye Res: 67, 383-393, 1998

[23] Heymer A: Aufbau eines dreidimensionalen humanen Cornea-Äquivalents, Diplomarbeit, Technische Universität Berlin, 2001

[24] Elbert KJ, Schäfer UF, Schäfers HJ, Kim KJ, Lee VHL, Lehr CM: Monolayers of human alveolar epithelial cells in primary culture for pulmonary absorption and transport studies.Pharm Res: 16, 601 – 608, 1999

[25] Evans MDM, Steele JG: Multiple attachment mechanisms of corneal epithelial cells to a polymer – cells can attach in the absence of exogenous adhesion proteins through a mechanism that requires microtubules. Exp Cell Res: 233, 88-98, 1997

[26] Hazlett L, Masinick S, Mezger B, Barrett R, Kurpakus M, Garrett M: Ultrasturctural, immunohistological and biochemical characterization of cultures mouse corneal epithelial cells. Ophthalmic Res: 28, 50-56, 1996

[27] Steele JG, Johnson G, Griesser HJ, Underwood PA: Mechanisms of initial attachment of corneal epithelial cells to polymeric surfaces. Biomaterials: 18, 1541-1551, 1997

[28] Hackworth LA, Faraji-Shadan F, Schuschereba ST, Bowman PD: Serum-free culture of porcine and rabbit corneal epithelial cells. Curr Eye Res: 9, 919-923, 1990

[29] Engelmann K, BohnkeM: Growing human corneal endothelium in cell culture. Fortschr Ophthalmol: 86, 72-75, 1989

[30] Reichl S, Muller-Goymann CC: The use of a porcine organotypic cornea construct for permeation studies from formulations containing befunolol hydrochloride. Int J Pharm: 250, 191-201, 2003

[31] Kawakita T, Espana EM, He H, Yeh LK, Liu CY, Tseng SC: Calcium-induced abnormal epidermal-like differentiation in cultures of mouse corneal-limbal epithelial cells. Invest Ophthalmol Vis Sci: 45, 3507-3512, 2004

Chapter 3

Comparative evaluation of corneal epithelial cell culture systems

for the assessment of ocular permeability

Parts of this chapter have been submitted to ATLA as:

Ulrich Becker, Carsten Ehrhardt, Marc Schneider, Leon Muys, Dorothea Groß, Klaus Eschmann, Ulrich F. Schaefer, Claus-Michael Lehr (2006) Comparative evaluation of corneal epithelial cell culture systems for the assessment of ocular permeability.

Abstract

Purpose: To evaluate different corneal epithelial cell culture systems for ocular drug permeability *in vitro* studies.

Methods: Transformed human corneal epithelial cells (HCE-T) and Statens Serum Institut rabbit cornea cells (SIRC) were cultured on permeable filters. Skinethic human corneal epithelium (S-HCE) and Clonetics human corneal epithelium (C-HCE) were received as ready-to-use systems. Excised rabbit (ERC) and human cornea (EHC), mounted in Ussing chambers, were used as references. Barrier properties were assessed by measurement of transepithelial electrical resistance and apparent permeability (P_{app}) of markers with different physico-chemical properties: fluoresceine-sodium, propranolol-hydrochloride, moxaverine-hydrochloride, timolol-hydrogenmaleate, and rhodamine123.

Results: SIRC cells and the S-HCE failed to develop barrier properties and hence, were unable to distinguish between the markers. Barrier function and power to differentiate permeabilities were found in HCE-T and even more pronounced in C-HCE. The latter corresponded very well with EHC and ERC. Net secretion of rhodamine123 was not observed in any model, suggesting that P-glycoprotein or similar efflux systems are unlikely to affect corneal permeability.

Conclusion: Currently available corneal epithelial cell culture systems show differences in epithelial barrier function. Those systems lacking functional cell-cell contacts are of limited value for assessing corneal permeability, but may be useful for other purposes, such as irritancy tests.

Introduction

The assessment of corneal penetration is crucial both for the development of ophthalmic medicines as for the safety evaluation of new materials. Until recently animal testing [1, 2, 3] has been the main option for such purposes, but an increasing number of *in vitro* models have already been suggested as potential alternatives[4-10]. Moreover, corneal constructs have been set-up, containing epithelial, stroma- and endothelial equivalents to mimic the whole cornea [11, 12]. However, a comparative evaluation of the currently available models has not yet been performed.

The reduction of animal experiments by such *in vitro* models is in accordance with the 3 R principles, firstly introduced by Russell and Burch in 1959 [13]. The 3 R's stand for **R**eplace, **R**educe and **R**efine and are guidelines for the application of animal experiments in scientific research. For the sake of animal welfare, cost reduction and a wish for enhanced reproducibility of experimental data, replacement of animal experiments by suitable *in vitro* test systems has become important issue.

Skinethic reconstituted human coreal epithelium (S.HCE, Skinethic), transformed human corneal epithelial cells from Araki-Sasaki's lab (HCE-T) [5], Statens Serum Institut rabbit cornea (SIRC) [9], and Clonetics human corneal epithelium (C-HCE, Cambrex) were selected for testing and comparison versus excised (native) human and rabbit cornea. The already mentioned corneal constructs represent very complex systems, truly mimicking the *in vivo* situation. A drawback of these systems is the complexity. Due to their three-layer-setup they are difficult to produce and take quite a long time to grow, which makes them only frequently available. Thus, we did not include these models. Still, the epithelial cell line used by Zorn-Kruppa *et al* [12] was the HCE-T cell line, which is included in our portfolio. The CEPI 17 CL4 cell line used by Reichl *et al* [11] is tightly regulated by its manufacturer and so unavailable for broad usage.

To evaluate the potential of these models to distinguish between drugs with respect to their corneal permeability, the transepithelial electrical resistance (TEER) was routinely measured to obtain an estimate of epithelial barrier function. Complementary histological data at light microscopy level were also obtained for all *in vitro* model tested. Subsequently, the following compounds with different physico-chemical properties were selected for transport experiments: fluorescein-sodium as a hydrophilic low permeability marker; propranolol-HCl as a lipophilic high permeability marker, rhodamine 123 as a substrate of P-glycoprotein to detect the possible activity of such efflux transporters. Additionally, we examined timolol-hydrogenmaleate and moxaverine-HCl as two drugs of ophthalmologic relevance [14].

Materials and Methods

Chemicals

Fluorescein-sodium (FluNa), rhodamine 123 (Rh123), formaldehyde and ethanol were obtained from Sigma (Seelze, Germany). Cyclosporine A (CyA) and penicillin/streptomycin were purchased from Calbiochem (Darmstadt, Germany). Propranolol-hydrochloride (Prop) was from Synopharm (Barsbüttel, Germany). Timolol-hydrogenmaleate (Tim) was a generous gift from Ursapharm Arzneimittel (Saarbrücken, Germany). ^{14}C-moxaverine-hydrochloride (Mox) was purchased from Biotrend (Cologne, Germany). Permeable filter inserts (Transwell 3460) were from Corning (Bodenheim, Germany). Eosin G 0.5% solution, Mayer's hematoxylin solution, xylene, Paraplast paraffin beads and Roti-Histokitt mounting reagent were from Carl Roth (Karlsruhe, Germany). All other chemicals were of highest commercially available grade.

Cell culture systems and tissues

Statens Serum Institut rabbit corneal (SIRC) epithelial cells: The SIRC cell line (ATCC CCl-60) was derived from the cornea of a normal rabbit in 1957 by Volkert at the Statens Serum Institut (Copenhagen, Denmark). Little is recorded relating to the history of this cell line for approximately the first 400 passages. It was originally used for the cultivation of rubella viruses. In 1990, the cells were morphologically characterized by Niederkorn and co-workers [15]. The cells do not exhibit an epithelial, cobblestone-like morphology, but a fibroblast phenotype. Cells were cultured in minimum essential medium (MEM) with 10% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin G. To stimulate growth, the cell culture medium was altered according to Hutak et al [9].

HCE-T cells (HCE-T): The HCE-T cell line was established in 1995 by Araki-Sasaki and co-workers [5]. It was gained by transfection of human corneal epithelial cells from a 47-year old

female donor with a recombinant SV40-adenovirus.HCE-T cells were purchased from Riken Cell Bank (RCB1384, Ibaraki, Japan) and cultured on Transwell filter inserts at a seeding density of 60,000 cells/cm^2 in DMEM/Ham's F12 1:1 mixture supplemented with 5% FBS, 5 µg/mL insulin, 0.1 mg/mL cholera toxin, 10 ng/mL EGF, 0.5% DMSO, 100 µg/ml streptomycin and 100 U/ml penicillin G. Cells of passage numbers 75 to 84 were used. A modified protocol according to Toropainen and co-workers [16] was used. In brief, cells were maintained for 1 week under liquid covered conditions and were then switched to air-interfaced condition in the second week. Medium was changed every other day during the first week and daily in the second week.

Skinethic reconstituted human corneal epithelium (S-HCE): Reconstituted human corneal epithelium was purchased from Skinethic (RHC/S/5, Nice, France). The model consists of human corneal epithelial cells immortalized by Beuerman at the Louisiana State University Eye Center (New Orleans, USA). S-HCE cells are routinely cultured by the manufacturer on NUNC filter inserts (CC-Inserts, 137052) and shipped to the end user at day 5 after seeding. Upon arrival, cells were transferred from the transport medium to the culture medium supplied by Skinethic. S-HCE were used within 24 hours of their arrival.

Clonetics cultured corneal epithelium (C-HCE): C-HCE were purchased from Cambrex (CMS-2015, Vervier, Belgium). The model is made from normal human corneal tissue. Epithelial cells were isolated and infected with an amphotropic recombinant retrovirus containing HPV-16 E6/E7 genes to extend the lifespan. The cells (age: day 20) arrived in Transwell filter inserts (3470) and were transferred to the provided medium upon arrival. C-HCE were used within 24 hours of their arrival.

Excised rabbit cornea: Rabbit corneas from animals to be slaughtered for food produce were bought from local breeders. No animals were sacrificed exclusively for our experiments. Eyes from pigmented purebred rabbits were enucleated directly after slaughtering. After removing adhering tissue, the cornea was obtained by cutting the scleral tissue in about 2 mm distance to the limbus between cornea and sclera. The cornea was cleaned by washing with PBS and then stored in cell culture medium (DMEM/F12 Ham 1:1 mix with 100 µg/ml streptomycin , 100 U/mL penicillin G, 100 µg/mL, insulin-transferrin-selenium premix, 30 µg/mL bovine pituitary extract , 1 ng/mL epidermal growth factor, 0.36 µg/mL hydrocortisone) until use.

Excised human cornea: Corneas considered not suitable for transplantation due to their low density of endothelial cells were donated by the Lions Cornea Bank (Homburg, Germany) and served as reference. Since the main permeability barrier of the cornea is represented by the corneal epithelium and the contribution of stroma and endothelium to the barrier properties of the cornea is neglectable [17, 18], this appeared to be acceptable for our purposes.

All cells and tissues were kept in an incubator at 37°C, 5% CO_2, 90% rH until use.

Histology

Filter-grown tissue samples were fixed in a formaldehyde solution (4% in PBS) at room temperature for 30 min. HCE-T and C-HCE samples were stained with hematoxylin for about 20 min. Four to 5 steps of washing with water stopped the staining process. Samples were then dehydrated through a graded series of ethanol at 70%, 96%, and 100% at room temperature and kept in xylene until embedding. For mounting, samples were soaked in paraffin wax at 60°C overnight and embedded the next day. Embedded samples were cut in four-micrometer thin sections with a microtome (Leica Microsystems, Nussloch, Germany) and mounted on glass slides at room temperature. Samples of human cornea and rabbit cornea

were counter-stained with Eosin G for 30 s and washed with ethanol of 96% and 100%. Samples were dewaxed with xylene and were rehydrated by a graded series of ethanol at 100%, 96%, and 70%, for 10 min each, at room temperature. The sections were washed with de-ionized water and staining was intensified by a 5 min treatment with Mayer's hematoxylin solution. Sections were stored in xylene until mounting using Roti-Histokitt. The sections were examined with an Axiovert 40 light microscope (Carl Zeiss, Jena, Germany) at 400x magnification.

For immunofluorescence staining of the nucleus, specimens were fixed with 2% paraformaldehyde (10 min) and subsequently blocked for 10 min in NH_4Cl solution (50 mM), followed by permeabilization for 15 min with 0.1% Triton X-100. Propidium iodide (1 µg/ml) was used to stain cell nuclei. After 30 min of incubation, the specimens were washed three times with PBS and embedded in FluorSafe anti-fade medium. Images were obtained by a confocal laser scanning microscope (MRC-1024, Bio-Rad, Hemel Hempstead, UK) with the instrument settings adjusted so that no positive signal was observed in the channel corresponding to green fluorescence of the isotypic control and the focus pane at the level of the nuclei of the topmost cell layer.

Transepithelial electrical resistance

To monitor the formation and functional integrity of cell layers the transepithelial electrical resistance (TEER) was measured with and epithelial voltohmmeter equipped with STX-2 "chopstick" electrodes (EVOM, World Precision Instruments, Berlin, Germany) and corrected for the background provided by the blank filter. Only cell layers with TEER values >400 $\Omega \cdot cm^2$ were used for the transport experiments. This value is generally accepted as the minimum requirement for tight barrier properties [19].

Transport studies

Transport of marker compounds was studied at the following concentrations: FluNa: 50 µM; Prop: 50 µM; Rh123; 13 µM; Tim 200 µM; Mox 33 µM = 1µCi/mL). Data are presented as mean ± standard deviation (*n*), where *n* is the number of observations.

For transport studies the cell layers were equilibrated for 1 h in modified Krebs Ringer buffer (KRB, 1.5 mM K_2HPO_4, 3 mM KCl, 4 mM glucose, 142 mM NaCl, 10.07 mM HEPES, 1.4 mM $CaCl_2$, and 2.56 mM $MgCl_2$, pH 7.4). The transport experiments were initiated by exchanging KRB of the respective donor side with the same volume of KRB containing the respective drug. The apical and basolateral volumes were 520 µL and 1520 µL (HCE-T) and 210 µL and 810 µL (S-HCE and C-HCE), respectively. To determine the initial concentration, 10 µL (S-HCE, C-HCE) and 20 µL (HCE-T) samples of the donor solution were assayed. Samples were drawn from the corresponding receiver compartment every 30 min for up to 4 h. Sampled volumes, i.e.,200 µL,(HCE-T) and 100 µL (S-HCE and C-HCE) were replaced by the same amount of fresh pre-warmed KRB. Transport of Rh123 was also determined in the presence of 10 µM CyA to determine the possible inhibitory efficacy on Rh123 transport.

Corneal tissues, either human or rabbit, were mounted in a modified Ussing chamber (round opening, 10 mm diameter) [20] and equilibrated with KRB for 1 h prior to the transport experiments. Flux studies were initiated by exchanging the KRB of the respective donor side with a same volume of marker solution. The volumes in the mucosal and serosal chamber were 2.5 mL each. Samples (200 µL) were drawn from the receiver compartment every 30 min for up to 4 h. Sampled volumes were replaced by the same amount of fresh pre-warmed KRB. To determine the initial donor concentration, 20 µL samples of the donor solution were assayed.

Sample analysis

For FluNa and Rh123, fluorescence activity of samples was analyzed in 96-well plates using a plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively.

For Prop and Tim, concentrations were determined by HPLC on a Summit system comprised of a P580 pump, ASI100 automated sampler, and UVD170S UV/VIS detector (Dionex, Idstein, Germany). A LiChroCART 125-4 LiChrosphere 100 RP-18 (5 µm) column was used and the samples were analyzed at 215 nm (Prop) or 297 nm (Tim), respectively. A mixture of acetonitile:methanol:water 22:33:45 with 330 µL triethylamine and 440 µL phosphoric acid served as mobile phase at a flow rate of 1.2 mL/min. Chromatograms were analyzed by estimating the area under the peak employing a computerized data integration program (Chromeleon 6.50, Dionex). Samples were diluted with KRB, where appropriate.

Mox was assayed with a liquid scintillation counter (Perkin Elmer, Rodgau, Germany) after mixing the samples with 4 mL Ultima Gold scintillation cocktail (Perkin Elmer).

The apparent permeability of the marker substances was calculated as

$$P_{app} = \frac{J}{c_0 * A} \qquad \text{(Eq. 1)}$$

where J = marker flux (mol/s), c_0 = initial marker concentration in the donor solution (mol/cm^3) and A = surface area of the corneal tissue and cell layers. The latter was 1.27 cm^2 for intact tissues, 1.13 cm² for HCE-T cell layers, 0.5 cm² for S-HCE, and 0.33 cm² for the C-HCE.

To quantify the power of the different models to distinguish between high and low permeability compounds, we calculated the high-low (h/l)-ratio,

$$h/l\ ratio = \frac{P_{app}(\mathrm{Pr}\,op)}{P_{app}(FluNa)} \qquad \text{(Eq. 2)}$$

Moreover, the permeability of all markers was compared for each model by calculating relative P_{app} values as a percentage of the high permeability marker propranolol.

Results

Histology

While excised human cornea and excised rabbit cornea are composed of all corneal cell layers, i.e., epithelium, stroma, and endothelium (Figure 1 A&B), the cell lines obviously consist only of epithelial cells (Figure 1 C-F). Apart from some differences in the size and shape of individual cells, most striking differences were found in the number and total thickness of the epithelial cell layers of the investgated models. Human cornea shows 4 to 5 epithelial layers, while we found a thicker epithelium for the rabbit cornea and a denser stroma.

While a progress in differentiation from basal cells to superficial cells can be clearly seen in excised human cornea, HCE-T cells (on day 14) maintain their cobblestone like appearance across all layers of a multilayer of 4-6 cells in thickness (Figure 1 C). CLSM images of propidium iodide-stained human cornea and HCE-T specimens underpin the difference in cell density and morphology when focused at the topmost cell layer (Figure 2 A&B). HCE-T cells grow to cuboidal-shaped cells at a very high density (Figure 2 B), while epithelial cells of the human cornea appear wider spread in shape (Figure 2 A).

The SIRC cell line has formed 4 cell layers on day 21. The fibroblast-like phenotype can be recognized in Figure 1 D. The difference in morphology is in accordance with the missing barrier properties, as shown by TEER measurements(see below).

The C-HCE model resembles the morphology of the rabbit tissue with a higher number of cell layers than the human counterpart (Figure 1 E). The epithelial cells exhibit various states of differentiation with cuboidal cells in the lower layers and cells of flattened appearance at the topmost aspect.

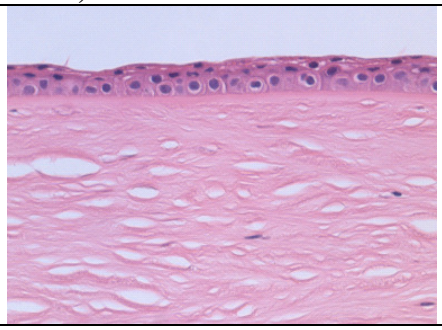
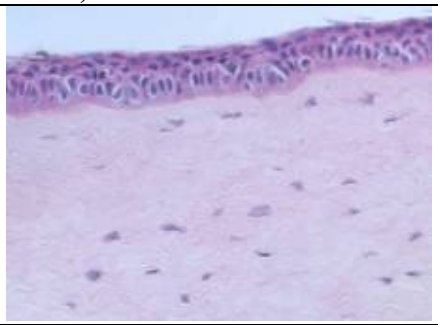
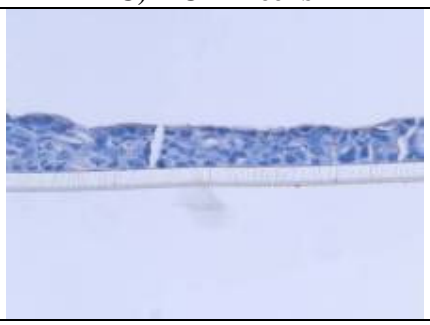
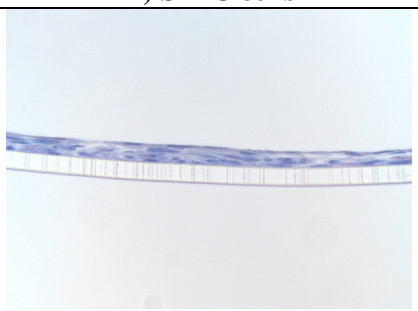
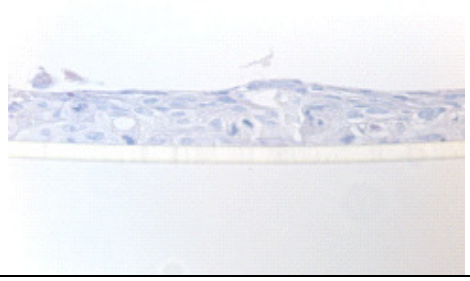
A) Excised human cornea	B) Excised rabbit cornea
	
C) HCE-T cells	**D) SIRC cells**
	
E) Clonetics human corneal epithelium	**F) Skinethic human corneal epithelium ***
	

Figure 1. *Histological examination of the different in vitro models by hemotoxylin-eosin staining. A) Excised human cornea; B) excised rabbit cornea; C) HCE-T cells; D) SIRC cells; E) Clonetics human corneal epithelium (C-CHE); F) Skinethic human corneal epithelium (S-CHE). Magnification 400x. * = Picture provided by Skinethic, France.*

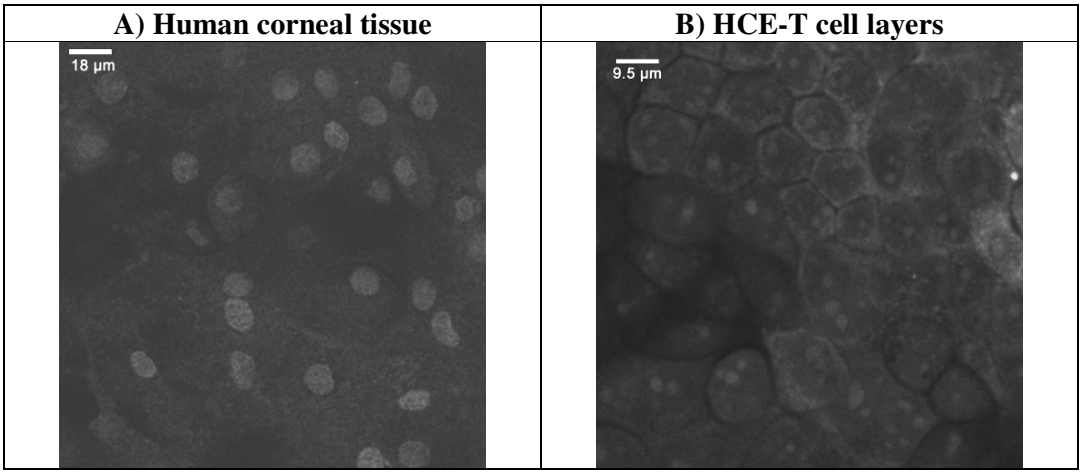


Figure 2. *Confocal laser scanning microscopy of A) and human corneal tissue and B) HCE-T cell layers. Images were obtained by focusing on the topmost cell layer Scale bars represent micrometers.*

Multiple cell layers can also be observed in the S-HCE model (Figure 1 F). Seven to 10 layers in thickness, the S-HCE cells are of cuboidal shape and only the most apical layer contains a number of cells with flattened morphology. Nevertheless, the S-HCE cell model does not show a clear differentiation of the cells with progressing layer number. Compared to the other cell systems, the number of layers is the highest, however the cells do not form tight junctions, as found by TEER measurements (see below).

TEER and permeability of transport markers

Formation of electrically tight cell layers is probably most important feature of an *in vitro* model of drug absorption. Therefore, TEER values were recorded for all models under investigation. In case of the in house grown cell lines, TEER was measured every time the medium was changed. The excised tissues and commercially available models were assessed

upon their arrival. The permeability of all marker compounds as well as typically observed TEER values for each of the tested models are summarized in Table 1.

Table 1: *Permeability values (P_{app}) of marker compounds across in vitro models of corneal epithelium and transepithelial resistance (TEER) of those models. All values are mean ± SD with n≥3*

Model	P_{app} (×10^{-6} cm/s)								TEER ± SD (Ω*cm^2)
	FluNa		Prop		Tim		Mox		
	AB	BA	AB	BA	AB	BA	AB	BA	
EHC	0.37 ± 0.26	0.57 ± 0.19	12.40 ± 2.65	8.24 ± 1.13	3.15 ± 1.03	2.50 ± 1.02	5.42 ± 1.97	3.76 ± 1.54	478 ± 355
ERC	0.12 ± 0.03	0.14 ± 0.04	5.63 ± 2.81	3.25 ± 1.01	3.52 ± 0.71	2.06 ± 0.50	5.16 ± 0.11	2.39 ± 0.71	848 ± 553
HCE-T	2.37 ± 0.29	2.29 ± 0.09	22.88 ± 5.33	14.99 ± 1.59	10.85 ± 1.91	9.03 ± 0.17	13.42 ± 0.83	17.49 ± 2.36	474 ± 120
S-HCE	6.96 ± 0.55	7.70 ± 0.53	14.78 ± 1.72	28.32 ± 3.19	9.10 ± 1.07	12.63 ± 0.32	15.49 ± 1.33	26.28 ± 3.10	106 ± 15
C-HCE	0.25 ± 0.10	0.32 ± 0.10	8.29 ± 0.20	7.36 ± 0.85	2.81 ± 0.40	1.65 ± 0.36	5.84 ± 0.27	6.82 ± 2.24	832 ± 365

Intact cornea, either human (EHC) or rabbit (ERC), which may be supposed to represent the most complete barrier showed clear differences in P_{app} values for the marker compounds. The lipophilic high permeability marker Prop showed P_{app} values in the range of 10^{-5} cm/s. Such values were reported earlier for other cell systems [19, 21, 22] and are thus in the range that is expected. In contrast, the hydrophilic marker FluNa showed low permeability, as indicated by a low P_{app} value in the range of $0.3x10^{-6}$ cm/s. Such values have also been reported earlier [6, 16]. A higher permeability than for FluNa was found for Tim and an almost equally high permeability as for Prop was found for Mox, corroborating the excellent ocular penetration of this compound *in vivo*, as was reported earlier[14]. Compared to EHC, ERC yielded the same ranking for the different P_{app} values which, however, were still approximately one magnitude lower than for EHC.

While SIRC cells did proliferate when cultured on permeable Transwell filters, the TEER remained at a level of the blank filter inserts (data not shown), suggesting that the cells did not develop tight junctions. As this could not be achieved by various alterations of the cell culture conditions, SIRC cells were not further considered for transport experiments

A low TEER of 100 $\Omega*cm^2$ was measured for the pre-grown cell layers of the Skinethic model. Nevertheless performed transport experiments yielded comparatively high P_{app} values even for the low permeability marker FluNa.

HCE-T cells formed tight cell layers, as indicated by a TEER of 500 $\Omega*cm^2$ and thus proved suitable for drug transport studies. P_{app} values were in the same magnitude as for EHC, but yielding results approximately twice as high as for EHC. Especially for the lipophilic markers, the power of differentiation was less than in EHC. Nevertheless, ranking of the substances with HCE-T was in accordance with the ranking found in EHC.

C-HCE cell layers were received as tight epithelium and correlated well to the values found for EHC. Not only the hydrophilic markers were found at identical P_{app} values of about $2x10^{-7}$ cm/s, but also the lipophilic markers were ranked identically in the range of 10^{-5} cm/s. Thus, correlation of this cell culture system to the intact human tissue (*ex vivo*) in terms of marker transport was very good.

P-glycoprotein-mediated efflux

Permeability of Rh123, a known substrate of P-glycoprotein, was assessed in both apical-to-basolateral (AB) and basolateral-to-apical (BA) direction. A BA/AB ratio of 2 or was not

observed in any of the *in vitro* models (Table 2). Moreover, addition of the P-gp inhibitor cyclosporine A (CyA) did not alter fluxes.

Table 2: *Bi-directional transport of the P-glycoprotein substrate rhodamine 123 (Rh123) across various in vitro models od corneal epithelium. Experiments were conducted with or without presence of the P-glycoprotein inhibitor cyclosporine A (CyA). Values are mean ± SD with n≥3*

Model	P_{app} (×10^{-6} cm/s)			
	Rh123		Rh123 + CyA	
	AB	BA	AB	BA
EHC	0.48 ± 0.48	0.59 ± 0.55	1.93 ± 1.37	1.16 ± 0.91
ERC	0.15 ± 0.09	0.26 ± 0.24	0.03 ± 0.02	0.06 ± 0.02
HCE-T	1.46 ± 0.42	2.25 ± 0.29	2.88 ± 0.11	4.39 ± 0.66
S-HCE	4.84 ± 0.37	6.42 ± 0.12	4.76 ± 0.24	5.70 ± 0.19
C-HCE	0.13 ± 0.01	0.26 ± 0.03	0.29 ± 0.05	0.22 ± 0.10

Power of differentiation

To compare the power of the various *in vitro* systems to differentiate between drug candidates, the ratio of P_{app} values for high and low permeability markers was calculated (Figure 3). In addition, the P_{app} values of the four permeability markers were normalized for each model as percentage of the permeability of the most permeable compound, Prop (Figure 4). The rank order of fluxes was Prop>Mox>Tim>FluNa, which is in agreement with decreasing lipophilicity. Significant difference between the models can be observed in the standard deviations of the normalized permeability ratios, as well as in the range between the highest (P_{app} (Prop) = 100% by definition) and lowest relative permeability (P_{app} (FluNa) between >60% and <5% of P_{app} (Prop)).

With an h/l ratio of nearly 40, EHC showed a good power of differentiation between the low and high permeability markers (Figure 3). The range of relative permeabilities spans from

100% to <5% with mostly non-overlapping standard deviations (Figure 4). For ERC, the h/l-ratio even exceeded the value for EHC. With an h/l-ratio of about 10, as well as a range of relative permeabilities between 100 and approx 15%, the HCE-T model clearly demonstrates a higher ability to differentiate compared to the S-CHE model. As can be seen from Figure 3, S-HCE had an h/l ratio of approx. 1, similar to cell-free filter membranes. The C-HCE model showed an h/l-ratio being the same as for the human cornea, but with by far the smallest standard deviations and broadest range for the relative P_{app} values.

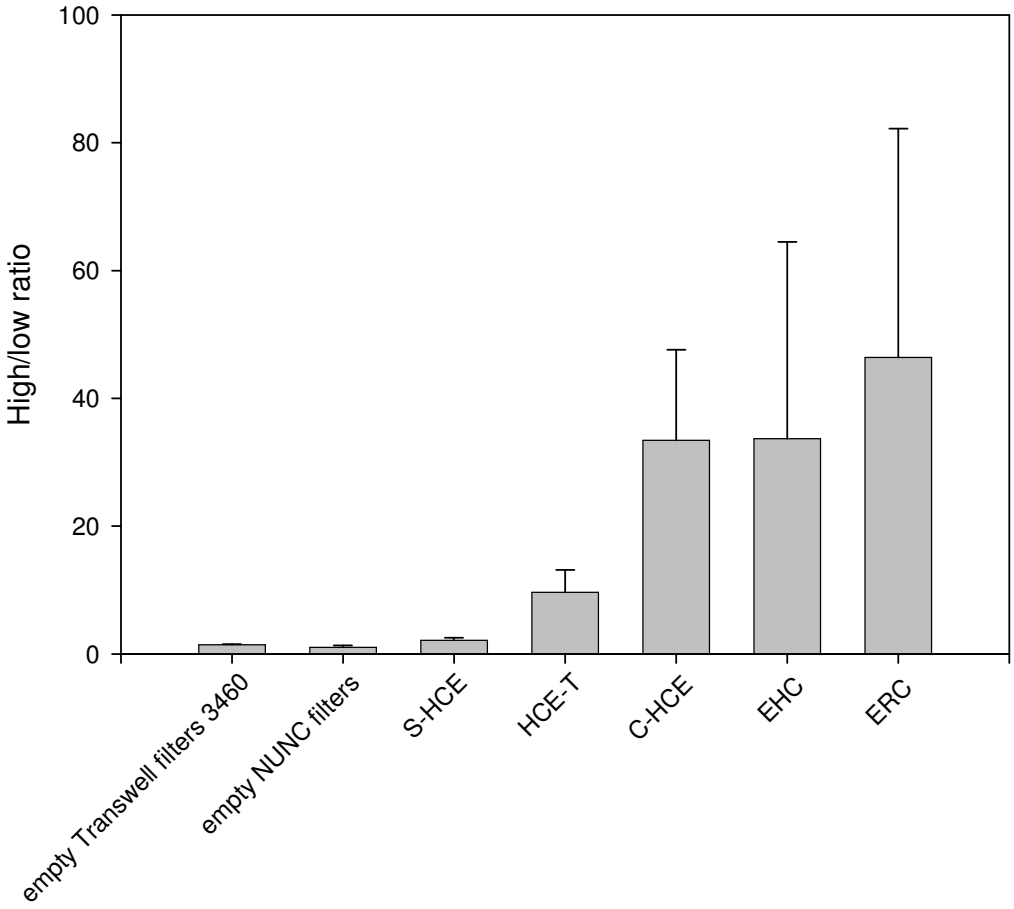


Figure 3. *Power of differentiation in marker permeability for the different in vitro models as expressed by calculating the respective high/low (h/l)-ratios (= P_{app}Prop/P_{app}FluNa). Data repesents mean ± SD, n = 3*

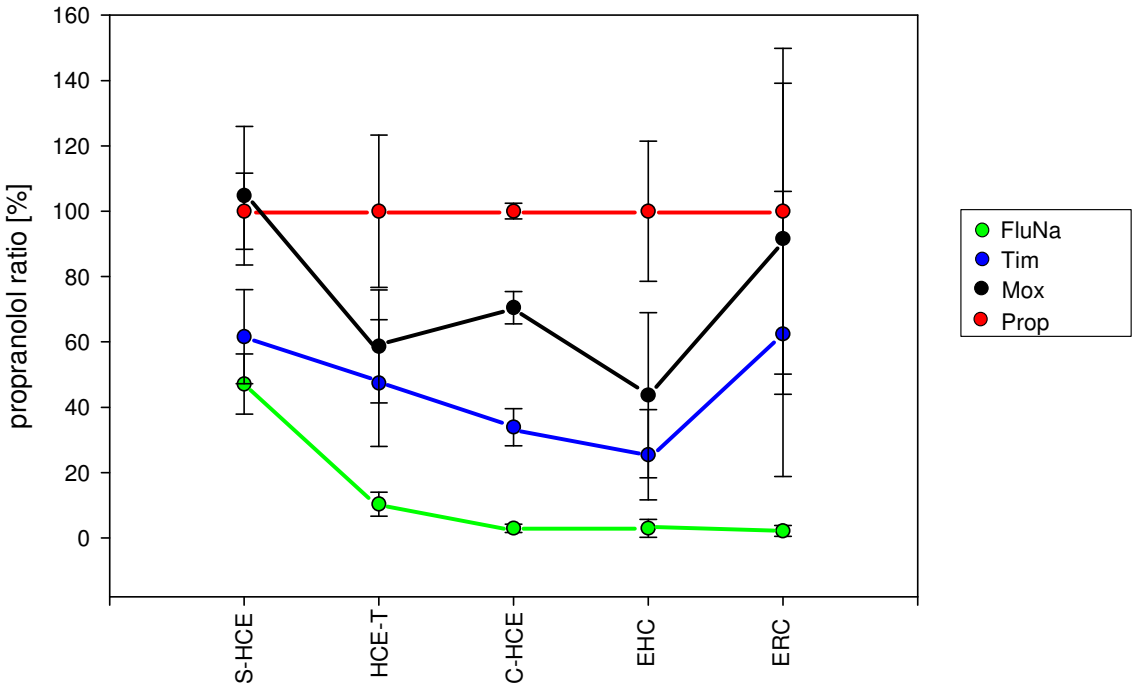


Figure 4. *Relative permeabilities of passive diffusion markers across the different in vitro systems. Values are shown as percentage of* P_{app} *Prop for each model, respectively. Error bars reflect the standard deviation by taking into account error propagation (i.e., summation of relative SD for both markers.)*

Discussion

In the presented work, we compared the currently available cell culture systems of corneal epithelium in their performance to assess permeability of xenobiotics. These models may help to reduce and replace animal experiments, by keeping handling and costs at a minimum, while providing robustness and good reproducibility. Still, the question arises, if such simple systems have sufficient power to differentiate and can be used as replacement for more complex ex vivo tissue from animals. Major progress was made in the replacement of the Draize test, hence, most of the available models are geared at this purpose, and barrier properties are typically not an issue. In this study, we used histology, bi-directional transport studies of passively transported drugs as well as P-gp substrates and the power of differentiation as tools to evaluate the models examined herein.

Due to inter-species differences excised rabbit cornea did not resemble the human cornea too closely. Morphological differences were obvious: the stroma seems to be of higher density and contains more keratinocytes and the epithelial cells grew to more layers than in the human tissue. These anatomical differences can be the cause for low fluxes of the low-permeability markers.

Two of the systems, the SIRC cell line and the S-HCE model disqualified at an early stage of the testing. Major drawbacks of these systems were the absence of tight cell layers.

Of the remaining models, the HCE-T cell line has been used in several published studies[16, 23]. HCE-T cells form a tight multi-layered epithelium with the ability to distinguish several marker substances, although the calculated P_{app} values were slightly higher than across human corneal tissue. However, drawbacks of the HCE-T model are found in histology; as HCE-T is an immortalized cell line, differentiation does not occur as completely as in human corneal epithelium. We noticed these differences by examining the cell lines by light microscopy / CLSM and comparing the pictures with EHC micrographs. HCE-T do not form completely

differentiated superficial cells, but maintains their cobblestone-like appearance to a certain degree. In addition, we previously reported on differences regarding efflux transporters which make this cell model rather unsuitable for drug permeation studies (Becker *et al.,* J Ocul Pharmacol Ther, in submission).

The C-HCE model provides tight cell layers with TEER values > 400 Ω·cm² and therefore appears as a useful model for permeation studies. The absolute P_{app} values of the marker compounds corresponded well with excised human cornea, while the reproducibility appeared to be even better. Together with the high power of differentiation these results make C-CHE a valuable model for *in vitro* permeability testing.

For reduction and replacement of animal experiments and refinement of methods, several *in vitro* models are available. Many are suitable as replacements for the Draize test. Of the models investigated in our study, C-CHE could be able to (at least partly) replace animal experiments to assess tissue distribution and penetration behavior of drug candidates.

Acknowledgements

Stephan Motz and Sebastian Tätz are thanked for their assistance with the HPLC analytics. Elisabeth Schmidt, Berlin and Frank Niedorf, Hannover are thanked for developing the Excel worksheet for data analysis.

References

[1] Draize JH, Woodard G, Calvery HO. Method for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Pharmacol Exp Ther* 1944;82:377–90.

[2] Pate DW, Jarvinen K, Urtti A, Jarho P, Jarvinen T. Effects of topical anandamines on intraocular pressure in normotensive rabbits. *Life Sci* 1996;58:1849-1860.

[3] Acheampong AA, Shackleton M, John B, Burke J, Wheeler L, Tang-Liu D. Distribution of brimonidine into anterior and posterior tissues of monkey, rabbit and rat eyes. *Drug Metab Dispos* 2002;30:421-429.

[4] Kahn CR, Young E, Lee IH, Rhim JS. Human corneal epithelial primary cultures and cell lines with extended life span: in vitro model for ocular studies. *Invest Ophthalmol Vis Sci* 1993;34:3429-3441

[5] Araki-Sasaki K, Ohashi Y, Sasabe T, Hayashi K, Watanabe H, Tano Y, Handa H. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci* 1995;36:614-621.

[6] Chang JE, Basu SK, Lee VHL. Air-interface condition promotes the formation of tight corneal epithelial cell layers for drug transport studies. *Pharm Res* 2000;17:670-676.

[7] Offord EA, Sharif NA, Mace K, Tromvoukis Y, Spillare EA, Avanti O, Howe WE, Pfeifer AM. Immortalized human corneal epithelial cells for ocular toxicity and inflammation studies. *Invest Ophthalmol Vis Sci* 1999;40:1091-1101.

[8] Mohan RR, Possin DE, Sinha S, Wilson SE. Development of genetically engineered tet HPV16-E6/E7 transduced human corneal epithelial clones having tight regulation of proliferation and normal differentiation. *Exp Eye Res* 2003;77:395-407.

[9] Hutak CM, Kavanagh ME, Reddy IK, Barletta MA. Growth pattern of SIRC rabbit corneal cells in microwell inserts. *J Toxicol - Cut Ocular Toxicol* 1997;16:145-156.

[10] Reichl S, Dohring S, Bednarz J, Muller-Goymann CC. Human cornea construct HCC—an alternative for in vitro permeation studies? A comparison with human donor cornea. *Eur J Pharm Biopharm* 2005;60:305-308.

[11] Reichl S, Bednarz J, Müller-Goymann CC. Human corneal equivalent as cell culture model for in vitro drug permeation studies. *Br J Ophthalmol* 2004;88:560-565.

[12] Zorn-Kruppa M, Tykhonova S, Belge G, Bednarz J, Diel HA, Engelke M. A human corneal equivalent constructed from SV40-immortalized corneal cell lines. *ATLA* 2005;33:37-45.

[13] Russell WMS, Burch RL: The Principles of Humane Experimental Technique, Charles Thomas Publishers, 1959 (online issue available at: http://altweb.jhsph.edu/publications/humane_exp/het-toc.htm)

[14] Becker U, Ehrhardt C, Schaefer UF, Gukasyan HJ, Kim KJ, Lee VH, Lehr CM. Tissue distribution of moxaverine-hydrochloride in rabbit eye and plasma. *J Ocul Pharmacol Ther* 2005;21:210-216.

[15] Niederkorn JY, Meyer DR, Ubelaker JE, Martin JH. Ultrastructural and immunohistological characterization of the SIRC corneal cell line. *In Vitro Cell Dev Biol* 1990;26:923-930.

[16] Toropainen E, Ranta V, Vellonen K, Palmgrén J, Talvitie A, Laavola M, Suhonen P, Hämäläinen KM, Auriola S, Urtti A. Paracellular and passive transcellular permeability in immortalized human corneal epithelial cell culture model. *Eur J Pharm Sci* 2003;20:99-106.

[17] Huang HS, Schoenwald RD, Lach JL. Corneal penetration behavior of beta-blocking agents II: assessment of barrier contributions. *J Pharm Sci* 1983;72:1272-1279.

[18] Huang AJ, Tseng SC, Kenyon KR. Paracellular permeability of corneal and conjunctival epithelia. *Invest Ophthalmol Vis Sci* 1989;30:684-689.

[19] Ehrhardt C, Kneuer C, Fiegel J, Hanes J, Schaefer UF, Kim KJ, Lehr CM. Influence of apical fluid volume on the development of functional intercellular junctions in the human epithelial cell line 16HBE14o-: implications for the use of this cell line as an in vitro model for bronchial drug absorption studies. *Cell Tissue Res* 2002;308:391-400.

[20] Grass GM, Sweetana SA. In vitro measurement of gastrointestinal tissue permeability using a new diffusion cell. *Pharm Res* 1988;5:372-376.

[21] Prausnitz MR, Noonan JS. Permeability of cornea, sclera, and conjunctiva: a literature analysis for drug delivery to the eye. *J Pharm Sci* 1998;87:1479-1488.

[22] Asano T, Ishihaa K, Morota T, Takeda S, Aburada M. Permeability of the flavonoids liquiritigenin and its glycosides in licorice roots and davidigenin, a hydrogenated metabolite of liquiritigenin, using human intestinal cell line Caco-2. *J Ethnopharmacol* 2003;89:285-289.

[23] Ranta VP, Laavola M, Toropainen E, Vellonen KS, Talvitie A, Urtti A. Ocular pharmacokinetic modeling using corneal absorption and desorption rates from in vitro permeation experiments with cultured corneal epithelial cells. *Pharm Res* 2003;20:1409-1416.

Chapter 4

Comparison of ABC-transporter expression in excised human cornea and the transformed cell line HCE-T

Parts of this chapter have been submitted to J Ocul Pharmacol Ther:

Ulrich Becker, Carsten Ehrhardt, Nicole Daum, Christiane Baldes, Ulrich F. Schaefer, Klaus W. Ruprecht, Kwang-Jin Kim, Claus-Michael Lehr (2006) Expression of ABC-transporters in human corneal tissue and the transformed cell line HCE-T

Abstract

Purpose: To compare expression profiles of P-glycoprotein (P-gp/MDR1), multi-drug resistance-associated protein 1 (MRP1), multi-drug resistance-associated protein 2 (MRP2), lung resistance-related protein (LRP), and breast cancer resistance protein (BCRP) in human cornea and the cell line, HCE-T.

Methods: Functional evidence for transporter activity was gathered by bi-directional flux studies across excised human cornea and HCE-T cell layers using rhodamine 123 (Rh123). Moreover, human cornea and HCE-T cells were examined for mRNA and protein expression of P-gp/MDR1, MRP1, MRP2, LRP, and BCRP, using RT-PCR and immunofluorescence microscopy.

Results: Flux studies of Rh123 revealed a slight but not significant ($p < 0.05$) asymmetry in transport across human corneas and HCE-T layers. Addition of cyclosporine A did not alter fluxes. Of all ABC-transporters under investigation, only LRP was found in human cornea. HCE-T cells did not show any signal for LRP, while expression of MRP1, MRP2, and BCRP could be confirmed. P-glycoprotein was not detected in any specimen under examination.

Conclusions: Human cornea expresses a very limited array of ABC-transporters. The expression pattern of HCE-T cells, however, differs from the native corneal tissue. Hence, this *in vitro* model should be used with caution to predict *in vivo* transport properties across the corneal epithelial barrier.

Keywords

Multi-drug resistance, ocular drug absorption, transport proteins, RT-PCR, *in vitro* models

Introduction

Topical eye medications are applied to the cul-de-sac of the eye, thus major emphasis of drug distribution and penetration is on the conjunctival-scleral pathway and the cornea. While the conjunctival-scleral pathway seems to be reserved for certain molecules of high molecular weight [1], the main transport pathway for the majority of drugs is represented by the cornea. The cornea consists of 3 regions with different physico-chemical properties: the 9 layers of the epithelium, which reflect the main barrier for drug penetration; the stroma; and an endothelial barrier. It has been found that only the epithelial cells exhibit tight junctions and adhere to each other via desmosomes [2], giving them important barrier properties. Stroma and endothelium on the other hand offer very little resistance to transcorneal permeation [3, 4]. The main function of the endothelium is to mediate ion and fluid transport and preserve the corneal transparency. The importance of the cornea as a penetration barrier raises the question of the presence of efflux transporter proteins as defense mechanisms, as well as obstacles for lipophilic drug penetration.

The proteins of the ABC (ATP-binding cassette) family have been recognized to limit the absorption of drugs across mucosal barriers and other tissues, such as the endothelium of the blood-brain barrier [5, 6]. The ABC-superfamily of transporter proteins represents mostly transmembranic moieties that promote efflux of xenobiotics and thus reduce their intracellular accumulation. Substrate binding is unspecific and covers a wide spectrum of substances [7]. Overexpression of these proteins has been found after continuous administration of substances to tumors, as well as healthy tissues, which earned these proteins the name “multi-drug resistance” (MDR) proteins. Since the cornea provides a major route for the drug penetration into the eye [8], it is likely that protective mechanisms are functionally expressed in the

cornea. However, information on the expression patterns of these ABC-transporters in human ocular tissues is scarce.

A variety of *in vitro* models based on excised tissues and epithelial cell cultures have been developed and refined for drug absorption/disposition studies over the last two decades. In this context, considerable attention has been given not only to the gastro-intestinal tract, but also to the development of *in vitro* models of ocular epithelial barriers [8 - 13, 26]. These efforts resulted in a number of continuous cell lines and cells in primary culture, which are now available to mechanistically study drug absorption across the ocular mucosa in a controlled environment. The human corneal epithelial cell line, HCE-T, is among these models. HCE-T cells were generated in 1995 by Araki-Sasaki and co-workers by immortalization of normal corneal epithelial cells from a 47-year old female donor using the SV40 virus [9]. HCE-T cell layers have been previously used as *in vitro* models for drug absorption experiments and toxicity testing [14, 15]. However, no data has been published on the array of transport proteins expressed by HCE-T to date.

In this study, transport experiments across whole excised human cornea and HCE-T cell layers were carried out to functionally confirm and compare the corneal expression of P-glycoprotein (P-gp/MDR1) using the substrate, rhodamine 123 (Rh123), and the inhibitor, cyclosporine A. Furthermore, excised human cornea and HCE-T cells were compared regarding the expression pattern of P-gp/MDR1, multi-drug resistance-associated protein 1 (MRP1), multi-drug resistance-associated protein 2 (MRP2), lung resistance-related protein (LRP), and breast cancer resistance protein (BCRP) at mRNA and protein level, using reverse transcription-polymerase chain reaction (RT-PCR) and immunofluorescence microscopy (IFM), respectively. In

addition, the morphology of the models in our hands was compared by light and confocal laser scanning microscopy (CLSM).

Materials and Methods

Materials

Cyclosporine A and penicillin/streptomycin were purchased from Calbiochem (Darmstadt, Germany). Rhodamine 123, mouse anti-P-gp antibody (clone F4), cholera toxin, bovine insulin, dimethyl sulfoxide (DMSO), formaldehyde and ethanol were from Sigma (Steinheim, Germany). Mouse anti-MRP1 antibody (clone MRPm6), mouse anti-MRP2 antibody (clone M_2 III-6), and mouse anti-BCRP antibody (clone BXP-21) were all purchased from Chemicon (Temecula, CA, USA). Rat anti-LRP antibody (clone LMR5) was from Alexis (San Diego, CA, USA), MDR primers were manufactured by MWG Biotech (Munich, Germany) with the sequences shown in Table 1. RNA isolation, reverse transcription and PCR were performed using an RNeasy Mini kit, Omniscript RT kit and Taq PCR Master Mix kit, all from Qiagen (Hilden, Germany). Permeable filter inserts (Transwell Clear, 12 mm diameter, pore size 0.4 µm) were from Corning (Bodenheim, Germany). Eosin G 0.5% solution, Mayer's hematoxylin solution, xylole, Paraplast paraffin beads and Roti-Histokitt mounting reagent were from Carl Roth GmbH & Co KG (Karlsruhe, Germany). All other substances used were of highest commercially available grade.

Tissue preparation and cell culture

Excised human cornea: Human corneas, unsuitable for transplantation due to their low density of endothelial cells, and leftover corneal rings from successful transplantations were a gift from the Lions Corneabank (University Hospital, Homburg, Germany).

HCE-T cells: HCE-T cells were purchased from Riken Cell Bank (Tsukuba, Japan). HCE-T cells (passage numbers 75 to 84) were cultured on Transwell Clear filter

inserts at a seeding density of 60,000 cells/cm^2. The culture medium consisted of Dulbecco's modified Eagle's Medium (DMEM)/Ham's F12 (1:1 mixture) supplemented with 5% fetal bovine serum (FBS), 5 µg/ml insulin, 0.1 mg/ml cholera toxin, 10 ng/ml epidermal growth factor, 0.5% DMSO, 100 µg/ml streptomycin and 100 U/ml penicillin G. A protocol communicated by Toropainen and co-workers [16] was slightly modified for our purposes. Briefly, cells were maintained for 1 week under liquid-covered conditions and were switched to an air-interface in the second week. Medium was changed every other day during the first week and daily in the second week.

Caco-2 cells: The Caco-2 cell line was used as a comparison for the expression of ABC-transporters. Cells were purchased from LGC Promochem (Wesel, Germany) and used at passage numbers 23 to 25. Caco-2 cells were cultured in flasks until they reached confluence on day 7 using DMEM supplemented with 10% FBS and 1% non-essential amino acids. The medium was changed every other day.

The development of cells into monolayers of epithelial morphology was monitored microscopically. In addition, cell growth and integrity of cell layers was monitored by measurement of the transepithelial electrical resistance (TEER). Only cell layers with TEER values >400 $\Omega \cdot cm^2$ were used for the transport experiments. The TEER values were assessed with an EVOM device equipped with STX-2 "chopstick" electrodes (World Precision Instruments, Berlin, Germany) and corrected for the background value of the blank filter.

Transport studies

Drug transport studies were performed with both HCE-T cell layers grown on Transwell Clear filter inserts for 14 days and excised human cornea mounted in modified Ussing chambers [17]. All solutions used were prewarmed to 37°C. Prior to transport studies, the cell layers/tissues were equilibrated for 1 h in modified Krebs Ringer buffer (KRB, 1.5 mM K_2HPO_4, 3 mM KCl, 4 mM glucose, 142 mM NaCl, 10.07 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), 1.4 mM $CaCl_2$, and 2.56 mM $MgCl_2$, pH 7.4). The transport experiments were initiated by exchanging the KRB of the respective donor side with the same volume of KRB containing rhodamine 123 (Rh123; 13 µM final concentration). The apical and basolateral volumes were 500 µL and 1500 µL in the Transwell system and 2.5 ml (both sides) in the Ussing chambers, respectively. Two hundred µL samples were drawn from the corresponding receiver compartment every 30 min for up to 4 h. Removed volumes were replaced by the same amount of fresh pre-warmed KRB. To determine the initial donor concentration, 20 µL samples of the donor solution, taken at t = 0, were assayed.

Fluorescence activity of Rh123 containing samples was analyzed in a 96-well plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively. These samples were diluted with KRB, where appropriate.

Unidirectional Rh123 fluxes were also determined in the presence of cyclosporin A (CyA), an inhibitor of P-gp, to determine the effect of CyA on Rh123 transport. In these inhibition studies, all solutions contained a final concentration of 10 µM CyA.

For human cornea, the identical experimental protocol was used. Human corneas were mounted in modified Ussing chambers, allowing access to front and rear side of the tissue. Donor and acceptor volumes were altered to 2.5 mL for both compartments according to the capacity of the respective sides. The defective endothelium and stroma were not considered a critical barrier in the transport experiments. Exposure to CyA to the basolateral side of the epithelium was achieved by a 1 h preincubation of the tissue with KRB containing 10 µM of CyA.

All experiments were performed in triplicates. For HCE-T cells, the same passage was used for all experiments. TEER values were assessed before and after the drug permeation experiments to ascertain the integrity of the corneas and the HCE-T cell layers.

The apparent permeability of Rh123 was calculated using the equation,

$$P_{app} = \frac{J}{c_0 * A} \qquad \textbf{(Eq. 1)}$$

where J = Rh123 flux (mol/s), c_0 = initial Rh123 concentration in the donor solution (mol/cm^3) and A = surface area of the corneal tissue or HCE-T cell layers, which was 1.27 cm^2 or 1.13 cm^2, respectively.

RNA isolation and RT-PCR

Corneal rings were separated from the scleral rim, cut into 10 mm^2 pieces and frozen in liquid nitrogen. Frozen tissues were then homogenized with an UltraTurrax homogenizer (IKA, Staufen, Germany). Subsequently, the nitrogen was evaporated and the residue was dissolved in lysis buffer (RNeasy kit). Further mincing of the corneal tissues was achieved by the use of a QiaShredder column (Qiagen). The lysate was purified for RNA according to manufacturer's instructions. For extraction of cellular proteins and RNA from Caco-2 and HCE-T cells in culture, cells were washed twice with phosphate-buffered saline (PBS, 129 mM NaCl, 2.5 mM KCl, 7.4 mM $Na_2HPO_4 \bullet 7\ H_2O$, 1.3 mM KH_2PO_4, pH 7.4) and then treated with trypsin/EDTA (0.25%/1 mM) for 5 min. Isolated cells were then extracted for RNA using QiaShredder columns and the RNeasy kit.

Reverse transcription was performed using the Omniscript kit in a Biometra Personal Cycler (Biotron GmbH, Göttingen, Germany) at 37ºC for 1 h followed by heating to 95ºC for 5 min. Polymerase chain reaction was conducted for 35 cycles, using the primers listed in Table 1 and a temperature program of 94ºC for 10 min initially, followed by 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1 min. The final step consisted of 72ºC for 10 min and storage of the end product at 4ºC. The resultant DNA fragments were examined by agarose (2%) gel-electrophoresis. GAPDH was used as an internal "loading" control.

***Table 1**. Primer sequences for PCR (for = forward primer; rev = reverse primer)*

Primer	Sequence
P-gp (for)	5'-CCCATCATTGCAATAGCAGG-3'
P-gp (rev)	5'-GTTCAAACTTCTGCTCCTGA-3'
MRP1 (for)	5'-ATCAAGACCGCTGTCATTGG-3'
MRP1 (rev)	5'-AGAGCAAGGATGACTTGCAGG-3'
MRP2 (for)	5'-CAAACTCTATCTTGCTAAGCAGG-3'
MRP2 (rev)	5'-TGAGTACAAGGGCCAGCTCTA-3'
LRP (for)	5'-CCCCCATACCACTATATCCATGTG-3'
LRP (rev)	5'-TCGAAAAGCCACTGATCTCCTG-3'
BCRP (for)	5'-TGCCCAGGACTCAATGCAACAG-3'
BCRP (rev)	5'-ACAATTTCAGGTAGGCAATTGTG-3'
GAPDH (for)	5'-CCCCTGGCCAAGGTCATCCATGACAACTTT-3'
GAPDH (rev)	5'-GGCCATGAGGTCCACCACCCTGTTGCTGTA-3'

Immunofluorescence microscopy

The respective antibodies were diluted 1:100 in PBS containing 1% (w/v) bovine serum albumin (BSA). For staining, corneal rings were cut into 10 mm^2 pieces as described above. The specimens were fixed for 30 min with 2% paraformaldehyde and excess paraformaldehyde was then removed by a treatment for 10 min in 50 mM ammonium chloride solution, followed by permeabilization for 15 min with 0.1% (w/v) Triton X-100. After 60 min incubation with 100 µL of the diluted primary antibody at 37°C, the corneal pieces were washed three times before incubation with 100 µL of a 1:100 dilution of a rabbit anti-rat IgG FITC-conjugate for LRP and a 1:100 dilution of Alexa Fluor 488-labeled goat anti-mouse IgG for all other proteins

in PBS containing 1% (w/v) BSA. Propidium iodide (1 µg/ml) was used to counterstain cell nuclei. After 30 min incubation, the specimens were washed three times with PBS and embedded in FluorSafe anti-fade medium. A negative control included the use of a mouse $IgG_{1,\kappa}$ (Sigma, Steinheim, Germany) and the specimen treated according to the described procedure. Images were obtained with a confocal laser-scanning microscope (MRC-1024, Bio-Rad, Hemel Hempsted, UK) with the instrument settings adjusted so that no positive signal was obtained in the negative control. IFM for HCE-T cells grown on Transwell filter inserts was performed using the same protocol as described above.

Statistical analysis

Results are expressed as mean ± S.E.M. Significance ($p < 0.05$) of differences in the group mean values for TEER and P_{app} were determined by one-way analysis of variances (ANOVA), followed by Student-Newman-Keuls post-hoc tests.

Results

Transport studies

The apparent permeability coefficients (P_{app}) of Rh123 are shown in Figure 1. For both, human cornea and HCE-T cells, no significant net directionality of Rh123 fluxes could be observed. The presence of CyA did not alter the flux ratios, but CyA increased the absolute permeability values in both directions for the HCE-T cell layers. The respective P_{app} values ($\times 10^{-7}$ cm/sec; mean ± S.E.M.; n = 3) for the different models/conditions were for human cornea, apical-to-basolateral (ab) 2.23±1.50 and basolateral-to-apical (ba) 7.15±2.96. The addition of CyA changed the ab value to 4.87±0.78; and the ba value to 7.89±3.56. HCE-T cell layers showed an ab-P_{app} value of 11.90±3.00 and ba value of 17.00±2.80. The addition of CyA altered the ab-P_{app} to 32.20±5.53 and 39.10±2.60 (ba).

RT-PCR

RT-PCR studies were carried out to assess the mRNA expression profiles for P-gp, MRP1, MRP2, LRP, and BCRP. As shown in Figure 2, in excised human cornea only LRP produced a positive signal. No message for any other ABC-transporter under investigation could be detected. In comparison, the immortalized HCE-T cells did not show a signal for LRP mRNA, whereas signals for MRP1, MRP2, and BCRP could be detected. Caco-2 cells, which were used as control, expressed all mRNAs except for LRP.

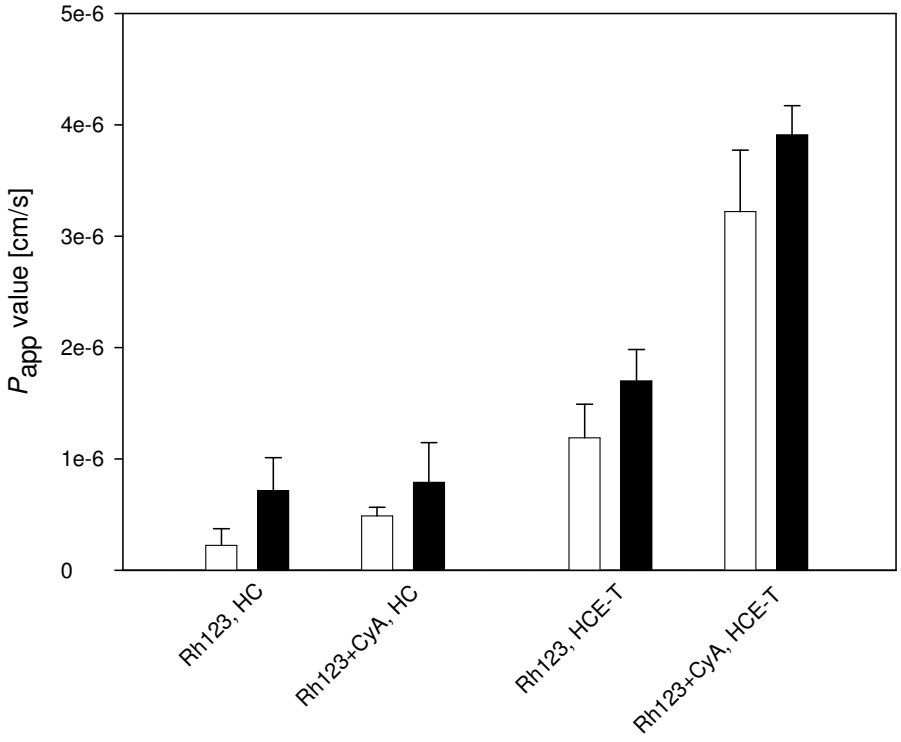


Figure 1. *Apparent permeability (P_{app}) of rhodamine (Rh123) across cultured layers of HCE-T human corneal epithelial cells (day 14) and excised human cornea. Excised human corneas were mounted in Ussing chambers and HCE-T cell layers were cultured on Transwell Clear filters for 14 days. Bi-directional transport studies (apical-to-basolateral (■) and basolateral-to-apical (□) directions) were carried out. No significant ($p < 0.05$) net directionality was found for Rh123 fluxes under all conditions tested. Presence of the P-glycoprotein inhibitor cyclosporine A (CyA) did not affect Rh123 flux ratios, but increased absolute fluxes across HCE-T cells. Data represent mean ± S.E.M. for n = 3 -4*

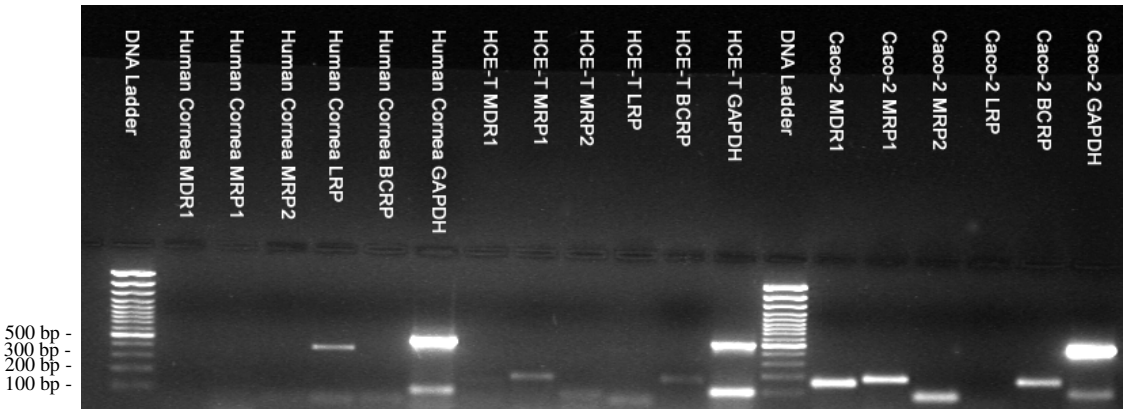


Figure 2. *Analysis of mRNA encoding for ABC-transporters by RT-PCR. The results for excised human cornea (lanes 2 to 6), HCE-T cells (lanes 7-12), and Caco-2 cells (lanes 14 to 19) are shown. GAPDH was used as an internal standard. Lane 1 shows the DNA ladder (ranging from 100 to 3000 bp). The bands for the amplicons are located on the gel at positions consistent with the expected sizes of 156 bp (P-gp/MDR1), 181 bp (MRP1), 56 bp (MRP2), 405 bp (LRP), 171 bp (BCRP), and 518 bp (GAPDH). In human cornea only the message for LRP was found, while HCE-T and Caco-2 cells contained mRNA encoding for MRP1, MRP2, and BCRP. Caco-2 cells, in addition, contained mRNA for P-gp.*

Immunofluorescence microscopy

The results of the IFM investigation are summarized in Figure 3. The presence of LRP in excised human cornea as a cytosolic vault protein was confirmed by IFM (Figure 3A). No signals for the other ABC–transporters under investigation could be observed by immunofluorescence (data not shown). IFM performed on Transwell-grown HCE-T cell layers resulted in data, which are in support of the findings from the RT-PCR studies (Figure 3B, C, D). LRP was absent (data not shown), while strong signals could be obtained for MRP1 (Fig. 3B), MRP2 (Fig. 3C), and BCRP

(Fig. 3D). However, neither human cornea nor HCE-T cell layers showed expression of P-glycoprotein at the cellular membranes (data not shown). The negative control with a mouse $IgG_{1,\kappa}$ did not show any staining (data not shown) and thus verified successful immunofluorescence staining.

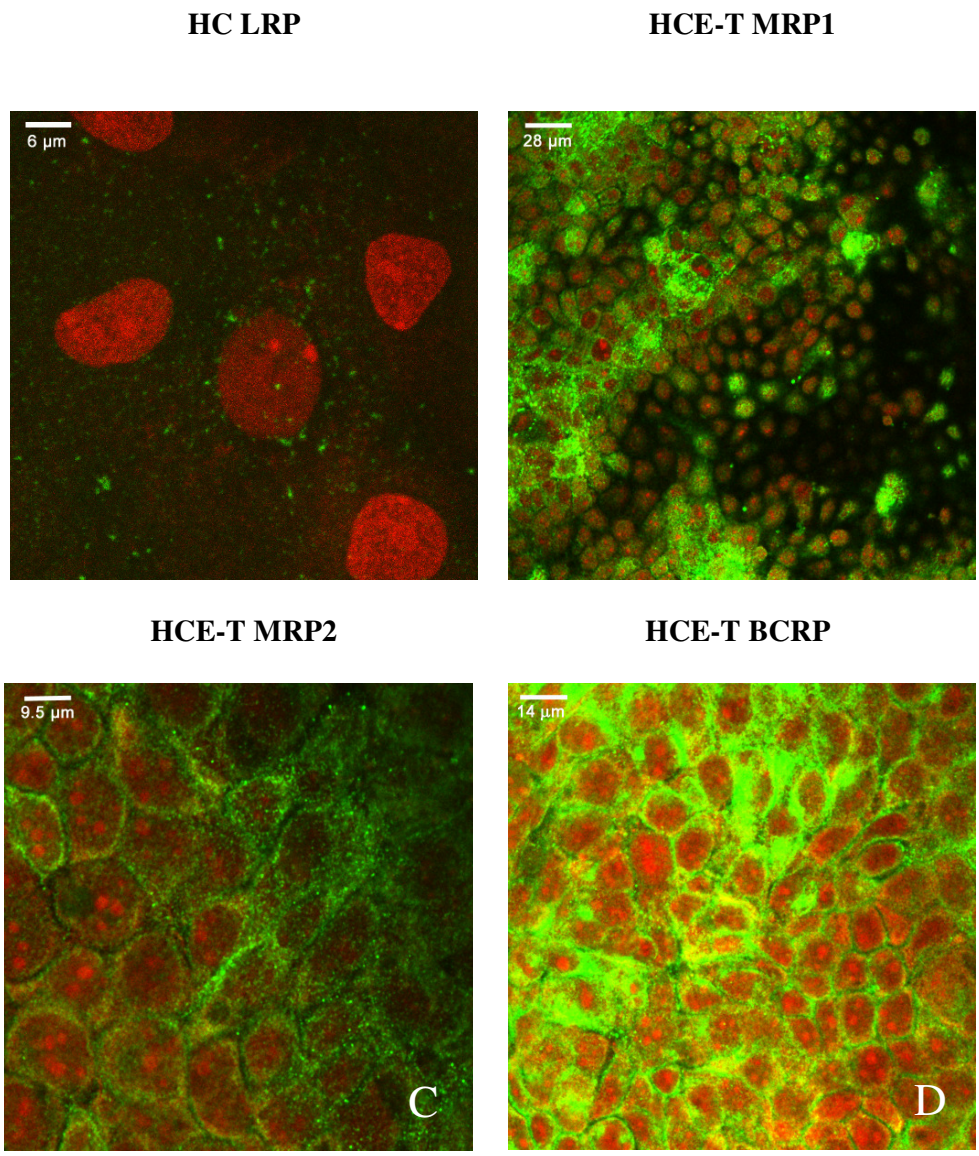


Figure 3. *Immunolabelling of ABC transporter proteins in excised human cornea and Transwell-grown HCE-T cell layers. Staining for proteins (green) is shown using confocal laser scanning microscopy. Nuclei were counterstained with propidium iodide (red). A: LRP in human cornea; B: MRP1 in HCE-T; C: MRP2 in HCE-T; D: BCRP in HCE-T cells.*

Discussion

In this study, we investigated the presence of ABC-transporters in excised human cornea and HCE-T cell layers by means of bi-directional drug transport studies and methods of molecular cell biology. By choosing rhodamine 123 as substrate we mainly concentrated on the examination of P-gp. While the transport studies revealed no evidence for any such efflux system, we subsequently compared the expression profiles of other ABC-transporters in native human corneal tissue at mRNA and protein levels to those in the immortalized cell line, HCE-T. Our results show that in HCE-T cells, LRP was not found; instead MRP1, MRP2, and BCRP were expressed, as assessed by semi-quantitative RT-PCR and IFM studies. The process of immortalization is generally regarded as a critical step. During the process of immortalization, the life span of the cells is extended and the growth characteristics are enhanced. This procedure generally decreases the state of differentiation. Therefore, it may not be too surprising that new features can be introduced into the cells and possible features of earlier states of differentiation can be reactivated [18]. For example, BCRP has been reported to be present in corneal epithelial stem cells, but is absent in the differentiated corneal epithelial cells [19].

In contrast, LRP was found to be expressed in human cornea. LRP is considered a ‘flag’ for the activation of an organic anion pump. LRP was also reported to be present in human tissues that are chronically exposed to xenobiotics, i.e., epithelial cells, supporting the assumtion that LRP is involved in the defense mechanisms of these cells [20]. It has been shown by Scherper and co-workers that LRP is a good drug-resistance-related predictor for the outcome of cancer treatments [21, 22], which makes these findings relevant for drug effect research. Due to its localization in the cytoplasm, effects of LRP on the Rh123 flux are not expected to affect the

directionality of transport across an epithelial cell layer, which is consistent with our findings.

In the primary cultured cells of rabbit conjunctiva, the presence of P-gp has previously been reported [23]. The transporter protein was shown to be mainly located in the apical part of the epithelial cell layer and found to be an important impeding factor for the absorption behavior of lipophilic drugs [24]. Other compartments of the mammalian eye for which a P-gp expression was reported, include lens (rat) [25], conjunctiva (rabbit) [23] and cornea (rabbit) [26, 27]. MRP1 has been reported for human retinal pigment epithelial cells [28], where it is functionally active.

Our findings are in contrast to a previously published study conducted by Dey and co-workers, who found evidence for the presence of P-gp/MDR1 mRNA in human corneal tissues [27]. While the difference in expression pattern between species is not entirely surprising, we have currently no explanation for the apparent discrepancy of their and our human data.

So far, corneal cell models have not been compared to intact human cornea. The only system tested against human tissue is the human cornea construct (HCC) developed by Reichl and co-workers [29]. The HCC system is a multilayered, three-dimensional construct, composed of three different cell lines and aiming to mimic the entire cornea. Thus, it is not surprising that a close resemblance between the relatively complex human cornea construct and native cornea for the tested markers was found. In the present study, however, we compared human cornea with the HCE-T cell line. The results show that there are significant differences in the expression pattern of drug efflux transporters between the native tissue and such a reductionist corneal epithelial model. In summary, in spite of being relatively easy to use, the HCE-T cell

line might be only of high value as an *in vitro* model for the examination of passive transport processes, as the preliminary examinations presented in this study indicate differences in the ABC-transporter expression.

Acknowledgements

The financial support from Ursapharm Arzneimittel GmbH & Co. KG is gratefully acknowledged. This work was supported in part by the National Institutes of Health Research grants HL38658, HL64365 and Hartings Foundation (KJK). Furthermore, Ms. Dorothea Gross and Dr. Klaus Eschmann (Ursapharm) are thanked for valuable discussions. The staff of the Lions Corneabank, Homburg is thanked for their supply of tissue samples. The authors also thank Dr. Marc Schneider for his help with the IFM pictures.

References

[1] Ahmed I, Patton TF: Importance of the noncorneal absorption route in topical ophthalmic drug delivery. *Invest Ophthalmol Vis Sci*. **26**: 584-587 (1985)

[2] Klyce SD, Crosson CE: Transport processes across the rabbit corneal epithelium: a review. *Curr Eye Res*. **4**: 323-331 (1985)

[3] Huang HS, Schoenwald RD, Lach JL: Corneal penetration behavior of beta-blocking agents II: assessment of barrier contributions. *J Pharm Sci*. **72**: 1272-1279 (1983)

[4] Huang AJ, Tseng SC, Kenyon KR: Paracellular permeability of corneal and conjunctival epithelia. *Invest Ophthalmol Vis Sci*. **30** 684-689 (1989)

[5] Takebayashi Y, Nakayama K, Fujioka T, Kanzaki A, Mutho M, Uchida T, Miyazaki K, Ito M, Fukumoto M: Expression of multidrug resistance associated transporters in porcine oocyte, *Intl J Molec Med*. **7**: 397-400 (2001)

[6] Verschraagen M, Koks CH, Schellens JH, Beijnen JH: p-glycoprotein system as a determinant of drug interactions: the case of digoxin-verapamil. *Pharmacol Res*. **40**: 301-306 (1999)

[7] Gottesman MM, Pastan I: The multidrug transporter, a doubleedged sword. *J Biol Chem*. **263**: 12163-12166 (1988)

[8] Kahn CR, Young E, Lee IH, Rhim JS: Human corneal epithelial primary cultures and cell lines with extended life span: in vitro model for ocular studies. *Invest Ophthalmol Vis Sci*. **34**: 3429-3441 (1993)

[9] Araki-Sasaki K, Ohashi Y, Sasabe T, Hayashi K, Watanabe H, Tano Y, Handa H: An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. **36**: 614-621 (1995)

[10] Chang JE, Basu SK, Lee VHL: Air-interface condition promotes the formation of tight corneal epithelial cell layers for drug transport studies. *Pharm Res*. **17**: 670-676 (2000)

[11] Offord EA, Sharif NA, Mace K, Tromvoukis Y, Spillare EA, Avanti O, Howe WE, Pfeifer AM: Immortalized human corneal epithelial cells for ocular toxicity and inflammation studies. *Invest Ophthalmol Vis Sci*. **40**: 1091-1101 (1999)

[12] Mohan RR, Possin DE, Sinha S, Wilson SE: Development of genetically engineered tet HPV16-E6/E7 transduced human corneal epithelial clones having tight regulation of proliferation and normal differentiation. *Exp Eye Res*. **77**: 395-407 (2003)

[13] Hutak CM, Kavanagh ME, Reddy IK, Barletta MA: Growth pattern of SIRC rabbit corneal cells in microwell inserts. *J Toxicol - Cut Ocular Toxicol*. **16**: 145-156 (1997)

[14] Toropainen E, Ranta V, Vellonen K, Palmgrén J, Talvitie A, Laavola M, Suhonen P, Hämäläinen KM, Auriola S, Urtti A: Paracellular and passive transcellular permeability in immortalized human corneal epithelial cell culture model. *Eur J Pharm Sci*. **20**: 99-106 (2003)

[15] Zorn-Kruppa M, Tykhonova S, Belge G, Bednarz J, Diehl HA, Engelke M: A human corneal equivalent constructed from SV40-immortalised corneal cell lines. *ATLA* **33**: 37-45 (2005)

[16] Toropainen E, Ranta V, Talvitie A, Suhonen P, Urtti A: Culture model of human corneal epithelium for prediction of ocular drug absorption. *Invest Ophthalmol Vis Sci*. **42**: 2942-2948 (2001)

[17] Grass GM, Sweetana SA: in vitro measurement of gastrointestinal tissue permeability using a new diffusion cell. *Pharm Res*. **5**: 372-376 (1988)

[18] Freshney IR: The transformed phenotype. In IR Freshney, Culture of animal cells, Alan R. Liss Inc, New York, 1987, pp. 199-208

[19] Schlötzer-Schrehardt U, Kruse FE: Identification and isolation of limbal stem cells. *Exp Eye Res*. **81**: 247-264 (2005)

[20] Mossink MH, van Zon A, Scheper RJ, Sonneveld P, Wiemer EAC: Vaults: a ribonucleoprotein particle involved in drug resistance? *Oncogene* **22**: 7458-7467 (2003)

[21] Izquierdo MA, Van der Zee AGJ, Vermorken JB, Van der Valk P, Beliën JAM, Giaccone G, Sceffer GL, Flens MJ, Pinedo HM, Kenemans P, Meijer CJLM, De Vries EGE, Scherper RJ: Drug resistance-associated marker LRP for prediction of response to chemotherapy and prognoses in advanced ovarian carcinoma. *J Natl Cancer Inst*. **87**: 1230-1237 (1995)

[22] List AF, Spier CS, Grogan TM, Johnson C, Roe DJ, Greer JP, Wolff SN, Broxterman HJ, Scheffer GL; Scherper RJ, Dalton WS: Overexpression of the major vault transporter protein lung-resistance protein predicts treatment outcome in acute myeloid leukemia. *Blood*. **87**: 2464-2469 (1996)

[23] Saha, P, Yang JJ, Lee VHL: Existance of a p-glycoprotein drug efflux pump in cultured rabbit conjunctival epithelial cells. *Invest Ophthalomol Vis Sci*. **39**: 1221-1226 (1998)

[24] Yang JJ, Kim KJ, Lee, VHL: Role of p-glycoprotein in restricting propanolol transport in cultured rabbit conjunctival epithelial cell layers. *Pharm Res*. **17**: 533-538 (2000)

[25] Merriman-Smith BR, Young MA, Jacobs MD, Kistler J, Donaldson PJ: Molecular identification of p-glycoprotein: a role in lens circulation? *Invest Ophthalmol Vis Sci*. **43**: 3008-3015 (2002)

[26] Kawazu K, Yamada K, Nakamura M, Ota A: Characterization of cyclosporin A transport in cultured rabbit corneal epithelial cells: p-glycoprotein transport

activity and binding to cyclophilin. *Invest Ophthalmol Vis Sci*. **40**: 1738-1744 (1999)

[27] Dey S, Patel J, Anand BS, Jain-Vakkalagadda B, Kaliki P, Pal D, Gnapathy V, Mitra AK: Molecular evidence and functional expression of p-glycoprotein (MDR1) in human and rabbit cornea and corneal epithelial cell lines. *Invest Ophthalmol Vis Sci*. **44**: 2909-2918 (2003)

[28] Aukunuru JV, Sunkara G, Bandi N, Thoreson WB, Kompella UB: Expression of multidrug resistance-associated protein in human retinal pigment epithelial cells and its interaction with BAPSG, a novel aldose reductase inhibitor. *Pharm Res*. **18**: 565-572 (2001)

[29] Reichl S, Dohring S, Bednarz J, Muller-Goymann CC: Human cornea construct HCC—an alternative for in vitro permeation studies? A comparison with human donor cornea. *Eur J Pharm Biopharm*. **60**: 305-308 (2005)

Chapter 5

Additional examinations

Introduction

In addition to the experiments already evaluated in Chapter 1 to 4, further experiments were performed. These include the light microscopical examination of the used cell models and a comparison of the efflux protein expression in human cornea and the permanent HCE-T cell line using Western blotting.

To evaluate the effect of the drug transport studies, the different *in vitro* models were evaluated microscopically after performance of transport experiments using a standard hematoxylin-eosin staining.

Models under investigation were human cornea, rabbit cornea, HCE-T cells grown on Transwell filter inserts for 14 days as well as the Statens Serum Institute Rabbit Cornea (SIRC) in different stages of cell development and the Cambrex Clonetics human corneal epithelial cell model.

The SIRC cell model was just examined for its growth behavior over 3 weeks because it does not exhibit tight junctions (shown by TEER measurements, values of ~ 90 Ω*cm² were recorded) and thus does not form a barrier significantly tight for drug transport.

All other models were examined in their "native" state before the experiments and after performing the transport studies. An effect of the various compounds was tried to evaluate.

To support the data on the gamut of ABC-transporter gamut in human cornea and HCE-T cells, immunoblotting experiments were performed. These experiments verified in part the data acquired through RT-PCR and immunofluorescence microscopy. Still, further experiments are needed to clarify the discrepancy between the immunoblotting data and the already shown data of chapter 4.

Materials and Methods

Materials

Sodium dodecylsulfate, mouse anti-P-gp antibody (clone F4), Quickdraw blotting paper, cholera toxin, bovine insulin, dimethyl sulfoxide (DMSO), formaldehyde and ethanol were from Sigma (Steinheim, Germany). Prestained wide-range protein markers were purchased from BioRad (Munich, Germany). Mouse anti-human MRP1 antibody (clone MRPm6), mouse anti-human MRP2 antibody (clone M_2 III-6), and mouse anti-human BCRP antibody (clone BXP-21) were all purchased from Chemicon (Temecula, CA, USA). Rat anti-human LRP antibody (clone LMR5) was from Alexis (San Diego, CA, USA), anti-mouse IgG alkaline phosphatase (AP)-conjugate and anti-rat IgG AP-conjugate were from Promega (Madison, WI, USA). Protran BA-85 nitrocellulose transfer membranes were purchased from Schleicher & Schuell (Dassel, Germany). Eosin G 0.5% solution, Mayer's hematoxylin solution, xylol, paraffin beads, Roti-Histokitt, Rotiphorese 30 acrylamide/bisacrylamide mixture, N, N. N', N' – tetramethylethylenediamine (TEMED), ammonium persulfate, glycine and tris-HCl were purchased from Roth (Karlsruhe, Germany). Complete mini protease inhibitor tablets and the AP substrate (NBT/BCIP) stock solution were from Roche Applied Sciences (Mannheim, Germany). Permeable filter inserts (Transwell Clear, 12 mm diameter, pore size 0.4 µm) were from Corning (Bodenheim, Germany). All other substances used were of highest grade available commercially.

Tissue preparation and cell culture

Excised human cornea: Non-transplantable human corneas, unsuitable for transplantation due to their low density of endothelial cells, and corneal rings were a gift from the Lions Corneabank (University Hospital, Homburg, Germany). Corneal rings were leftovers from successful cornea transplantations.

Caco-2 cells: The Caco-2 cell line was purchased from LGC Promochem (Wesel, Germany). Caco-2 cells (passages 23-25) were cultured in flasks using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% non-essential amino acids. The medium was changed every other day. The development of cells into monolayers of epithelial morphology was monitored microscopically. The cells were grown until they reached confluence, which was achieved after a 7-day period.

HCE-T cells: HCE-T cells were purchased from Riken Cell Bank (Tsukuba, Japan). HCE-T cells (passage numbers 75 to 84) were cultured on Transwell Clear filter inserts at a seeding density of 60,000 cells/cm². The culture medium consisted of DMEM/Ham's F12 (1:1 mixture) supplemented with 5% FBS, 5 µg/mL insulin, 0.1 mg/mL cholera toxin, 10 ng/mL epidermal growth factor, 0.5% DMSO, 100 µg/ml streptomycin and 100 U/ml penicillin G. As described earlier, cells were maintained for 1 week under liquid covered conditions and were switched to an air-interface condition in the second week. Medium was changed every other day during the first week and daily in the second week to contribute to the cell's high metabolic activity.

Clonetics corneal epithelial cell models: Clonetics cell culture models (Cat. No.: CMS-2015) were purchased from Cambrex (Vervier, Belgium). Upon arrival, cells were removed from the transport gel and transferred into the supplied 24-well cell culture plate. Cells were maintained under air-interface conditions with the provided, freshly supplemented cell culture medium (CEBM, Cat.No: CC-3251, supplemented with Corneal Epithelial SingleQuot kit, CC-4443). Maximum incubation time before the experiments was twelve hours.

Methods

Western blotting

For immunoblotting, three groups of cell lysates were generated. The human corneal rings (1.7 cm in diameter; n = 4) were transplantation leftovers and consisted of a scleral part of 3

mm width and a corneal part approximately 4 mm wide. The scleral rim was carefully removed using a pair of scissors and forceps. The remaining corneal tissue was cut in pieces of 13.5 mm^2 and frozen in liquid nitrogen, followed by homogenization using an UltraTurrax rotor-stator homogenizer. After evaporation of the nitrogen, the homogenized tissue was resuspended in 1 mL modified radioimmunoprecipitation (RIPA)-buffer with protease inhibitors (150 mM NaCl, 50 mM tris-HCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate, 5 µg/mL aprotinin, and 5 µg/mL leupeptin; pH 7.4). The solution containing tissue homogenates was kept on ice for 30 min and sonicated for 30 s every 10 min. After lysis, the suspension was centrifuged at 14,000 rpm (20,000×*g*) at 4ºC for 4 min. An aliquot of the supernatant was taken for determination of protein amount and the remainder frozen for storage until assay later. A BCA-assay (Uptima, St. Augustin, Germany) was used for protein quantification.

For the preparation of lysates from Caco-2 and HCE-T, cells were removed from the cell culture flask with trypsin/EDTA and washed with DMEM medium. The suspension of isolated cells was centrifuged and the cell pellet was resuspended in 1 mL RIPA-Buffer with protease inhibitors. The solution containing cells was kept on ice for 30 min and sonicated for 30 s every 10 min. The cell lysate was then centrifuged at 14,000 rpm at 4ºC for 4 min. An aliquot of the supernatants was taken for protein determination and the remainder frozen immediately until use.

For immunoblotting analysis, 20 µg each of the various protein lysates were diluted with reducing Laemmli's sample buffer (130 mM Tris-HCl, 6% sodium dodecylsulfate, 20% glycerol, 0.1% bromphenole blue, and 10% β-mercaptoethanol, pH 6.8) to make up 30 µL of loading volume in each lane. Samples were denatured at 56ºC for 10 min and pipetted into each lane of the gel pockets. Electrophoresis was performed using a Mini Protean II (BioRad) at 80 V for 10 min and then at 100 V for 80 min to fractionate the proteins by their molecular weights.

For detection of P-gp, MRP1, MRP2, and BCRP, a 6% polyacrylamide gel was used with the wide range marker cocktail loaded in one lane. For the detection of LRP and GAPDH (internal standard), a 10% gel was used with wide range protein markers. Fractionated proteins were then transferred onto nitrocellulose membranes at 48 V for 2 h. Successful protein transfer was checked by Ponceau staining. The membrane was achromatized and blocked over night with blocking buffer containing 10% low fat milk and 0.1% Tween 20.

Blocked membranes were incubated with the respective primary antibody diluted in blocking buffer for 90 min at room temperature. The membrane was washed for 2 min with PBS, twice with PBS containing 0.05% Triton X-100 for 5 min and again with PBS for 2 min. An appropriate AP-conjugated secondary antibody diluted in the blocking buffer was incubated with the membrane for 90 min at room temperature. After washing the membrane again as described above, the antibody-antigen complexes were detected with NBT/BCIP solution (Roche Applied Science, Mannheim, Germany).

Histological staining

A standard hematoxylin & eosin staining protocol was used. Briefly, cells were fixed in a formaldehyde solution (4% in PBS) at room temperature for 30 min. Excess formaldehyde was removed by repeated washing with water. Samples were then dyed with hematoxylin for about 20 min. The staining process was stopped by 4 to 5 washing steps with water. Washing was ceased after the washing liquid remained colorless. Samples were then dehydrated over night through a graded series of ethanol at 70%, 96% and 100% at room temperature and kept in xylol afterwards. For embedding, samples were soaked in paraffin wax at 60°C over night and embedded the next day.

Four micrometer sections were cut with a microtome (Leica Microsystems, Nussloch, Germany) and stored in xylol. Samples were rehydrated by a graded series of ethanol at 100%, 96% and 70% for 10 min each at room temperature. The sections were washed with

water and staining was intensified by another 5 min treatment with Mayer's hematoxylin solution. No further staining was performed with HCE-T cell layers. Tissue samples of human cornea and rabbit cornea were counter-stained with Eosin G for 30 s and washed with a series of ethanol 96% and 100%. Sections were stored in xylene until mounting on object slides with Roti-Histokitt. Paraffin wax embedded sections were examined with a Zeiss Axiovert light microscope. (Carl Zeiss, Jena, Germany)

Results

Protein expression

Expression patterns of ABC transporter proteins were investigated by means of Western blotting. The immunoblotting partly confirmed the results obtained at the mRNA level and by immunofluorescence microscopy (see Chapter 4). Excised human corneal tissue shows positive signals for LRP only (Figure 1), while HCE-T cell layers express MRP1 and BCRP, but not LRP (Figures 1 and 2). Caco-2 cells, as expected, express P-gp/MDR1, MRP1, MRP2, and BCRP.

Light microscopy

Micrographs of the intact tissues are shown in Figure 3. Thickness of the epithelial cell layer varies with the type of tissue. In human cornea (Figure 3A) only four to five epithelial cell layers are recognizable. The stroma shows its characteristically collagen structure with implemented fibroblasts. Rabbit cornea (Figure 3B) shows similar features to human cornea. The epithelial cell layer shows a higher number of cell sheets and a progression in differentiation. This progress is visible by the change in morphology.

HCE-T cells are only epithelial cells and therefore miss the stroma and endothelial cells. HCE-T cells on day 14 do not show much differentiation even though they grow in multiple layers (Figure 4A).

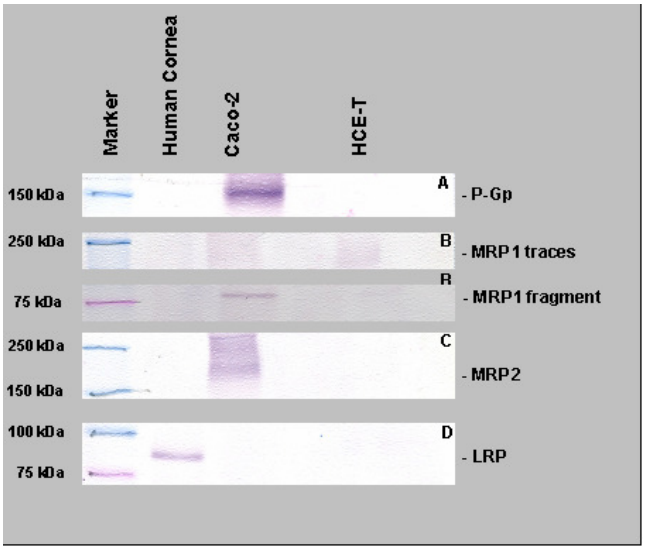


Figure 1. *Representative Western blots of P-gp, MRP1, MRP2, and LRP. Lane 2 shows protein lysate from the excised human cornea, lane 3 control cell lysate (from Caco-2 cells), and lane 4 contains HCE-T cell lysate. Western blots for each of the ABC-transporters are representative of at least 2 independent experiments.*

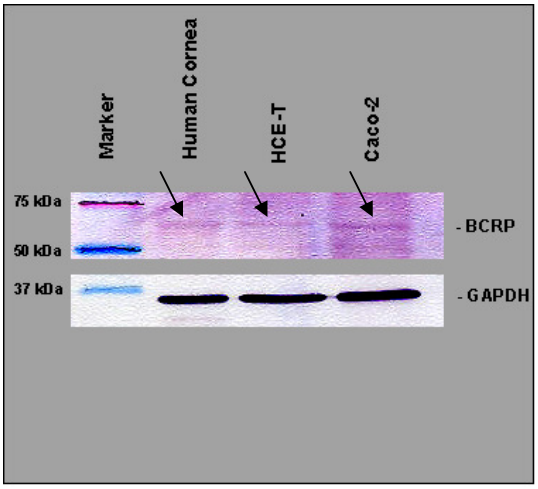


Figure 2. *Representative Western blots of BCRP and GAPDH (internal control). Lane 2 shows protein lysate from the excised human cornea, lane 3 contains HCE-T cell lysate, and lane 4 control cell lysate (from Caco-2 cells). Western blots are representative of at least 2 independent experiments.*

The Clonetics model is also a reductionist model and consists of epithelial cells only. The cells grow in multiple layers of about 6 cell layers. A progress in differentiation is clearly visible through changed morphology (Figure 4B).

The changes in model composition are shown in Figure 3 and 4. Changes are variable depending on the model and on the substance used. In general, it is visible that drug transport studies across corneal epithelium do affect the cell layers. In many cases, a disaggregation of the cells is visible. Also a swelling of the stroma can be detected in case of the human and the rabbit cornea.

The proliferation of SIRC cell layers is shown in Figure 5. Pictures of the cell sheets were taken on day 7 (Fig. 5A), day 14 (Fig. 5B) and day 21 (Fig. 5C)

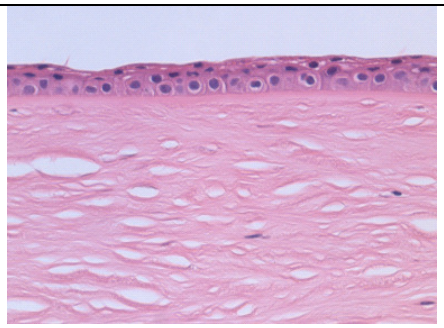
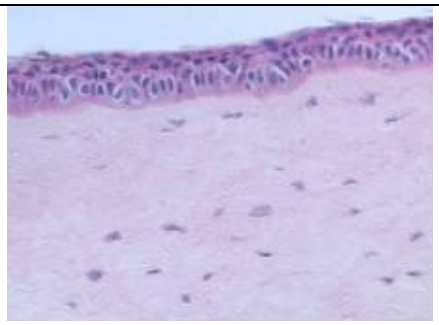
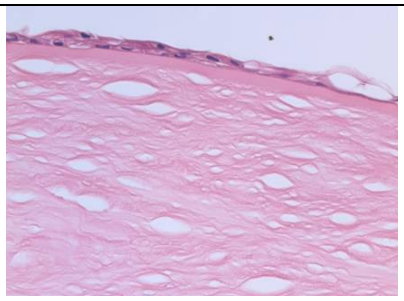
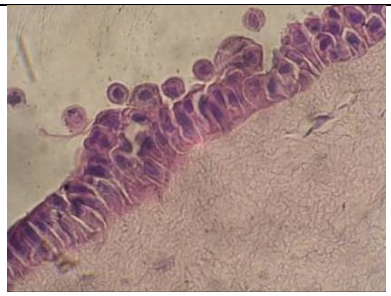
	Excised human cornea	**Excised rabbit cornea**
Before		
After		

Figure 3: Ex vivo *tissues before and after performance of transport experiments*

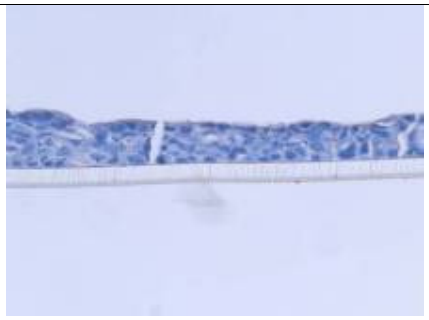
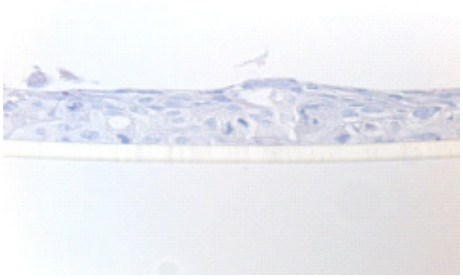
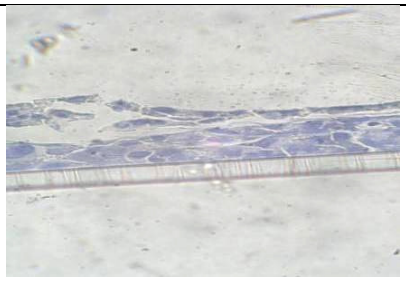
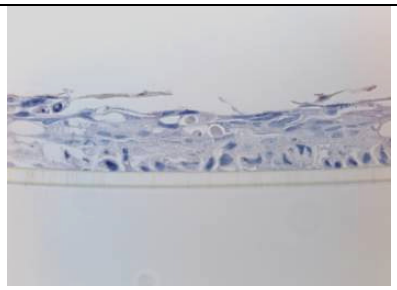
	HCE-T cells	**Clonetics human corneal epithelium**
Before		
After		

Figure 4: In vitro *cell layers before and after performance of transport experiments*

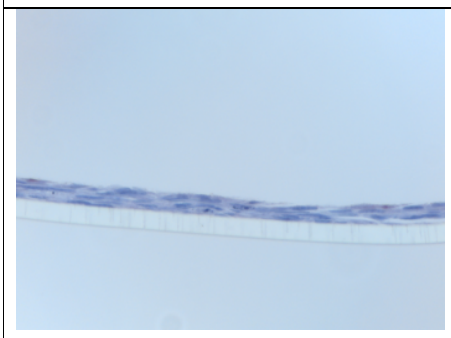
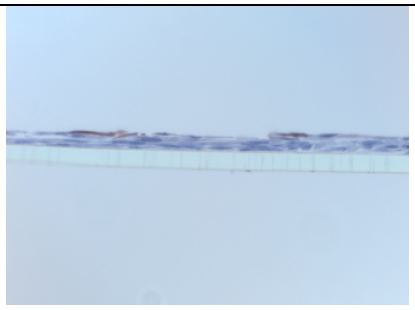
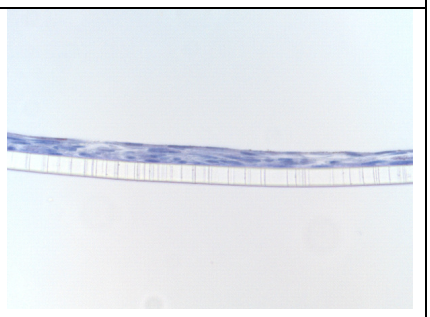
Day 7	Day 14	Day 21
		

Figure 5: *Development of SIRC cell layers cultured on Transwell filter inserts for 21 days*

Discussion

Western blotting confirmed the data acquired by RT-PCR and immunofluorescence microscopy. Human cornea shows only a positive signal for LRP and BCRP, which has been reported to be a corneal stem cell marker [1]. HCE-T cells therefore show a broader spectrum of ABC-transporter proteins. MRP1 and BCRP were found by immunoblotting. LRP did not show any signal, which is in accordance to the data acquired earlier. Surprisingly, MRP2 was not found by western blotting, even though we clearly proved its presence by RT-PCR and immunofluorescence microscopy. This discrepancy might be due to instability of the antibody-antigen-complex or insufficient sensitivity of the detection method. Further experiments have to be carried out to clarify this matter. In any case, a successful blotting process was verified by the internal standard/loading control GAPDH.

Differences in the morphology of the specimens have been detected by light microscopy. During drug transport studies, major changes occurred and were detected by post-transport light microscopy. In many cases, disaggregation of the cells and stroma were detected. In one case we also detected intensive signs of inflammation. If this inflammation was caused by the drug transport study or if the inflammation already existed prior to the experiment could not be determined in this experiment. Since ocular injuries often occur to breeded rabbits, it is reasonable to assume that the inflammation already existed at beginning of the experiment.

Major disaggregation was also detected in the HCE-T cell model indicating that these cell layers have an integrity shift during transport experiments. These findings are in accordance to the recorded TEER values, that usually show a mean drop of about 200 $\Omega*cm^2$.

Since the TEER values were still in the range of about 300 $\Omega*cm^2$ for all cell layers used in experiments, a lack of cell layer tightness is not probable. Still, changes in cell tightness and cell layer adherence are to be taken in account.

References

[1] Schlötzer-Schrehardt U, Kruse FE: Identification and characterization of limbal stem cells. Exp Eye Res: 81, 247–264, 2005

Conclusions, Summary and Outlook

Conclusions

The presented work gives a thorough overview on „state of the art“ *in vitro* cell cultures of the corneal epithelium.

The initial aim of this study, setting-up a primary corneal epithelial cell culture model based on human tissue, however, was not achieved. As other researchers in the field, we found that major obstacles, such as too little tissue amounts and poor viability of the cells in the corneal epithelium, circumvent the successful continuous culturing of these cells to tight monolayers. Recent advantages in cell isolation techniques are still to inefficient to promote cell accumulation for this specific tissue.

For the examination of the penetration behavior of moxaverine-hydrochloride (Mox), we found in different models (rabbit *in vivo*, primary rabbit corneal epithelial cell culture, human *ex vivo*, rabbit *ex vivo*, human *in vitro* corneal epithelial cell culture) unisonous data that cornea does not represent a critical barrier for the ocular delivery of this drug. However, the low solubility in aqueous solutions of physiological pH presents a major obstacle on the way to successfully market moxaverine-HCl as a topically-administered medicine.

In this thesis, I was able to evaluate a number of different *in vitro* models of the corneal epithelium. I found that those models that were mainly established as substitutes for the Draize test disqualify for drug transport studies due to their lack of functional cell-cell contacts. Nevertheless, these models are of high value for eye irritation assays. Systems that were developed with the aim to provide tight cellular layers (i.e., HCE-T and the Clonetics corneal epithelium model) are useful tools to assess passive corneal transport. However, for active transport, the HCE-T model should be used with caution, since it was found in the cause of my studies that the spectrum of ABC-transporter proteins significantly differs from native human cornea. The Clonetics human corneal epithelium should also be used with care,

because its ABC-transporter spectrum has not been assessed so far. The SIRC cell line does neither form tight cell layers nor matches the human corneal morphology and should thus not be used for drug transport studies at all.

Summary

The focus of my thesis was to find a simple test system to screen new therapeutic entities to regarding their ocular penetration. Using the model compound moxaverine-hydrochloride, the project was performed in 4 phases:

In phase 1, the tissue distribution of moxaverine-hydrochloride in the rabbit eye was examined and evaluated. A distribution profile was generated by either dosing pigmented Dutch-belted rabbits topically on the eye or applying the drug solution intravenously. Results showed that high drug amounts in the posterior part of the eye can be achieved after pre-corneal disposition of the moxaverine-solution. The fact that blood-plasma levels of moxaverine-hydrochloride did not exceed the levels found after systemic dosing makes local ocular administration of moxaverine-hydrochloride an interesting therapeutic approach. Parallel to the *in vivo* testing, the barrier that corneal epithelium exhibits towards moxaverine-hydrochloride was examined using a primary rabbit corneal epithelial cell culture (rbCECL). These *in vitro* drug transport studies confirmed that the rabbit cornea does not represent a critical barrier for moxaverine-penetration into the eye.

Phase 2 of the presented project dealt with the development of a primary human corneal epithelial cell model. Many attempts of creating such a model have been described frequently throughout the last decades. Even though scientists often succeeded in isolating cells and setting up primary cell cultures, cell isolation proved unreliable and cumbersome in all cases. Since significant progress has been made in recent years in our understanding of cell isolation and culture, applying these techniques to the human corneal epithelium seemed feasible. The methods mainly concentrated on various enzymatic digestions, using proteases and combination of these enzymatic techniques with a cell positive selection protocol using magnetic microbeads coated with human epithelial antigen (CD327, HEA-125). Even though

the experimental conditions were varied intensively and numerous combinations of enzymes were examined, the aim of the primary human corneal epithelial cell culture was not reached. The limited amount of available tissue and the long storage time of the corneal samples after excision might contribute to the failure of this part of the work.

Since a primary human corneal epithelium was out of reach as an *in vitro* test system, existing models of human corneal epithelium were examined and compared to intact human and rabbit cornea (phase 3 of the thesis). The cell models under investigation were all of human origin and immortalized by either SV-40 large T antigen or a recombinant retrovirus containing HPV16 genes E6 and E7. The cell models were examined by a spectrum of marker-substances for their power to differentiate (high/low permeability markers), presence and activity of P-glycoprotein efflux systems (using substrate molecules), and value for ophthalmic research ("ophthalmology marker"). The systems were also used to screen the substance of interest, moxaverine-hydrochloride.

Using a set of marker substances, a ranking of the models was created. The commercially available reconstituted human corneal epithelium from Skinethic (Nice, France) showed a poor performance in this study, even though it serves as a valuable tool in toxicity and eye irritation testing. The likewise commercially available Clonetics system from Cambrex as well as the long established HCE-T cell line proved to be good simplified models of the cornea. Drawbacks for the HCE-T cell line, however, is the less pronounced power of differentiation and the findings made in stage 4 of the project (see below).

In the final, fourth phase of the project, the HCE-T cell line was compared to the human cornea regarding its equipment with common efflux transporters. Since efflux systems of the ABC-transporter family are known to be a crucial factor in drug disposition and failure in cancer therapy, these proteins have gained major interest in biopharmaceutical research.

To clarify the situation in corneal epithelium that has not been extensively reviewed so far, we examined the presence of MDR1/P-gp, MRP1, MRP2, LRP, and BCRP in *ex vivo* human corneal samples and the commonly used HCE-T cell line. RT-PCR and immunofluorescence microscopy, as well as immunoblotting were used as tools to assess the spectrum of transporters.

Human cornea proved to be a rather uncritical tissue concerning multi-drug resistance issues, showing only a little array of efflux proteins, namely only LRP, HCE-T, however, showed a broader spectrum of efflux proteins, not unexpected in a continuously growing cell line that highly differs from the situation found *ex vivo*. These finding somewhat reduce the value of the HCE-T model in drug transport research.

Zusammenfassung

Die vorliegende Arbeit konzentrierte sich auf das Erforschen eines einfachen Testsystems, um das oculare Penetrationsverhalten neuer therapeutischer Substanzen vorhersagen zu können. Unter Verwendung der Modellsubstanz Moxaverin-Hydrochlorid wurde das Projekt in 4 Abschnitten durchgeführt:

In Phase 1 wurde die Gewebeverteilung von Moxaverin-Hydrochlorid im Kaninchenauge ermittelt und bewertet. Ein Verteilungsprofil wurde erstellt, indem pigmentierte Kaninchen der Rasse “dutch-belted” eine Wirkstofflösung entweder lokal am Auge oder intravenös verabreicht wurde. Die Resultate zeigten, daß hohe Substanzmengen im hinteren Teil des Auges nach precornealer Verabreichung der Moxaverinlösung erreicht werden können. Die Tatsache, daß die Blutplasma-Spiegel des Moxaverin-Hydrochlorids die der systemischen Gabe nicht überstiegen, macht die lokale, okulare Gabe von Moxaverin-Hydrochlorid zu einer interessanten Therapiemöglichkeit für die vom Koopertionspartner vorgesehene Anwendungsgebiete. Parallel zur *in vivo* Testung wurden die Barriereeigenschaften des Korneaepithels gegenüber Moxaverin-Hydrochlorids mit einer primären Epithelzellkultur der Kaninchenkornea (rbCECL) überprüft. *In vitro* Arzneistofftransportstudien zeigten, daß das Kaninchenhornhautepithel keine kritische Barriere für die Moxaverinpenetration in das Auge darstellt.

Phase 2 der vorgelegten Arbeit beschäftigte sich mit der Entwicklung eines primären humanen Korneaepithelzellmodells. Versuche, ein derartiges Modells zu etablieren, sind bereits mehrmals während der letzten Jahrzehnte beschrieben worden. Obwohl die Wissenschaftler häufig erfolgreich Zellen isolieren und Primärzellkulturen etablieren konnten, stellte sich die Zellisolation bisher stets als unreproduzierbar und mühsam heraus. Da sich die Techniken in den letzten Jahren deutlich weiterentwickelt haben und zahlreiche Protokolle z.

B. für primäre menschliche alveolare Zellekulturen erstellt wurden, erschien die Übertragbarkeit dieser Methoden auf das menschliche Korneaepithel möglich. Die angewandten Techniken bestanden hauptsächlich aus enzymatischen Verdau mittels verschiedener Proteasen, die bereits in der Literatur beschrieben wurden. Diese wurden mit einem Zell-Aufreinigungsverfahren kombiniert, das sich magnetischer Microbeads bedient. Diese sind mit einem menschlichem Epithelantigen (CD326, HEA-125) beschichtet und besitzen die Möglichkeit, spezifisch an humane Epithelzellen zu binden. Obwohl die experimentellen Bedingungen immer wieder variiert wurden und verschiedene Isolationstechniken kombiniert wurden, konnte das gesetzte Ziel der primären menschlichen Korneaepithelzellkultur nicht erreicht werden. Die begrenzte Menge des vorhandenen Gewebes und die lange Kulturzeit der Korneaproben nach Entnahme aus dem Spender trugen hierbei massgeblich zum Misslingen dieses Teils der Arbeit bei.

Da ein primäres menschliches Korneaepithel als *in vitro* Testsystem nicht verfügbar war, wurden bereits etablierte Modelle für das menschliche Korneaepithel mit intakter Menschen- und Kaninchenhornhaut verglichen (Abschnitt 3 des Projekts). Alle untersuchten Zellmodelle waren menschlichen Ursprungs und durch das SV-40 large T Antigen oder ein recombinantes Retrovirus, das die HPV16 Gene E6 und E7 enthielt, immortalisiert. Die Zellmodelle wurden mit einem Spektrum von Testsubstanzen auf ihre Differenzierungskapazität (hoher/niedriger Permeabilitätsmarker), das Vorhandensein von P-gp Effluxsystemen (mittels Substrattransportes) und Wert für die Augenforschung („Ophthalmologiemarker“) überprüft. Die Systeme wurden weiterhin als Screen für die Modellsubstanz, Moxaverin-Hydrochlorid, benutzt. Mittels dieser Markersubstanzen wurden die Zellmodelle klassifiziert. Das käuflich erhältliche rekonstituierte menschliche Korneaepithel von Skinethic (Nizza, Frankreich) zeigte nur eine niedrige Differenzierungsleistung, obwohl es ein wertvolles Testsystem im Bereich Augenreizung und Toxizitätsstudien darstellt. Das ebenfalls kommerziell erhältliche

Clonetics System von Cambrex und die etablierte HCE-T Zelllinie waren gute vereinfachende Modelle der Hornhaut. Die HCE-T Zelllinie zeigte allerdings weniger ausgeprägte Unterscheidungskraft als das Clonetics System und die Resultate die in Phase 4 des Projektes (siehe unten) gefunden wurden, führten zu einer weiteren Rückstufung des Modells.

In der letzten, vierten Stufe dieser Arbeit wurde die HCE-T Zelllinie mit der menschlichen Hornhaut bezüglich der wichtigsten bekannten Effluxsysteme verglichen. Da die Effluxsysteme der ABC-Transporterfamilie sich als entscheidender Faktor in der Arzneistoffabsorption über biologische Barrieren herausgestellt haben und auch eine entscheidende Rolle in der Resistenzbildung von Tumoren in der Krebstherapie spielen („mulit-drug resistance“), haben diese Proteine ein grosses Interesse in der biopharmazeutischen Forschung geweckt. Da es zum Expressionsmuster der ABC-Transporter in der menschlichen Hornhaut nur wenige und zudem widersprüchlich Aussagen gibt, wurde das Vorhandensein und die Aktivität von MDR1/P-gp, MRP1, MRP2, LRP und von BCRP in Humankornea mit in der vielgenutzten HCE-T Zelllinie verglichen. RT-PCR und Immunofluoreszenzmikroskopie, sowie Immunoblotting wurden hierbei als Methoden benutzt. Menschliche Hornhaut stellte sich hierbei als ein ziemlich unkritisches Gewebe bezüglich multidrug-resistance Proteinen dar und zeigte ein sehr eingeschränktes Spektrum an Effluxproteinen (LRP). HCE-T Zellen, stattdessen besitzen ein breites Spektrum von Effluxtransportern, das sich in hohem Grade von der *in vivo* Situation unterscheidet. Dies mindert deutlich den Wert dieser Zelllinie als *in vitro* Modell für die Erforschung von (aktivem) Arzneistofftransport.

Outlook

For the future, the picture on *in vitro* models of the human corneal epithelium still has to be refined. This work found good correlations for passive transport across some of the *in vitro* models which were able to give a clear rank order of the marker compounds under investigation, however, the question of active transport is still not completely elucidated. First examinations on human cornea and the HCE-T cell line have been performed, but do not cover the whole spectrum of efflux proteins. Neither have the other model systems been investigated in this regard.

Other topics that need to be addressed are the comparison of the cell culture models with the meanwhile available human corneal constructs

Based on recently published date these cornea constructs systems might be promising tools for the testing of drug formulations, safety testing/risk assessment of formulations, and questions of penetration enhancement and enhanced drug retention on the ocular surface.

Appendices

List of abbreviations

AIC	Air-interfaced culture
BCRP	breast cancer resistance protein
BSS	Balanced salt solution
C-HCE	Clonetics human corneal epithelium
CLSM	Confocal laser scanning microscopy
CyA	Cyclosporin A
DMEM	Dulbecco's minimum essential medium
DMEM/F12	1:1 mixture of DMEM and Ham's F12 medium
EDTA	(Ethylenedinitrilo) tetraacetic acid, disodium salt
EGF	Epidermal growth factor
EHC	Excised human cornea
ERC	Excised rabbit cornea
EVOM	Epithelial volt-ohm meter
FBS	Fetal bovine serum
FluNa	Fluorescein-sodium
HCE-T	SV40-transfected human corneal epithelial cells
HEPES	4-(2-Hydroxyethyl)-piperazine-1-ethansulfonic acid
KRB	Krebs-Ringer buffer
LCC	Liquid-covered culture
LRP	lung resistance-related protein
MDR	Multi-drug resistance

MEM	Minimal essential medium
Mox/MOX	Moxaverine-hydrochloride
MRP	multi-drug resistance-associated protein
NEAA	Non-essential amino acids
P-gp	P-glycoprotein
P_{app}	Apparent permeability
PBS	Phosphate buffered saline
PD	Potential difference
Prop	Propranolol
rbCrECL	Rabbit corneal epithelial cell layers
Rh123	Rhodamine 123
RT-PCR	Reverse transcription polymerase chain reaction
S-HCE	Reconstituted human corneal epithelium (Skinethic)
S-MEM	supplemented MEM
TEER	Transepithelial electrical resistance
Tim	Timolol-hydrogenmaleate

ULRICH BECKER

PERSONAL INFORMATION

Date of birth	:	January, 24th 1977
Place of birth	:	Erbach (Odenwald), Germany
Nationality	:	German

EDUCATION

1983 - 1987	Grundschule Hoechst Hoechst (Odenwald), Hessen
1987 - 1993	Ernst-Goebel-Schule, Hoechst Hoechst (Odenwald), Hessen
June 1993	*Mittlere Reife*
1993 - 1996	Berufliches Gymnasium Fachrichtung Wirtschaft Michelstadt (Odenwald), Hessen
June 1996	*High School Diploma*
1997 - 2001	School of Pharmacy, Johann-Wolfgang Goethe University Frankfurt am Main, Hessen
May 2002	*Pharmacist, Approbation*
Since Sept. 02	Saarland-University, Dept. Biopharmaceutics and Pharmaceutical Technology Saarbruecken, Saarland

PhD Student working on "Ocular cell models"

Since Sept. 02	Euro PhD Studies as project member of the "Galenos Network in the Pharmaceutical Sciences" PhD Cooperation with the *University of Southern California, School of Pharmacy:* Training in biopharmaceutical techniques, animal handling and in vivo studies. *Supervisors: Prof. Dr. C.M. Lehr (Saarland University) and Prof. Dr. V.H.L. Lee (University of Southern California)*

PROFESSIONAL MEMBERSHIPS

Association for Research in Vision and Ophthalmology (ARVO)

Acknowledgements

I would like to thank Prof. Dr. Claus-Michael Lehr for giving me the opportunity to work on this project and for his advice.

Furthermore, I would like to thank Prof. Dr. Hans H. Maurer for giving his expert opinion on my thesis.

Dr. Carsten Ehrhardt is thanked for co-supervising my thesis, his constant advice, for guiding me through the difficulties of this project and for his expert opinion on my thesis.

I also want to thank Dr. Ulrich F. Schaefer for co-supervising me, his helpful advice, sharing his ideas, reviewing my thesis and for pushing the project forward at the right time.

Vincent Lee, PhD. is thanked for taking me into his laboratory at USC, Los Angeles and providing me with space and material for my work.

Kwang-Jin Kim, PhD. is thanked for the many helpful discussions, repeatedly reviewing my manuscripts without getting fed-up with it and for giving me a good time in Los Angeles.

Ursapharm Arzneimittel GmbH&Co KG, for financially supporting my work and providing me with moxaverine-hydrochloride. Special acknowledgements are given to Dorothea Gross and Dr. Klaus Eschmann for the helpful discussions and guiding my work.

Acknowledgements

The staff at the Lions Corneabank, Homburg (Saar) is thanked for providing me with donor corneas and for their kind co-operation.

Special thanks go out to Dr. Stefan Reichl for introducing me to the world of ocular science, for being available for my questions at any time and finally giving me an helpful overview on corneal cell culture.

Dr. Nicole Daum and Dr. Christiane Baldes are thanked for introducing me in the secrets of molecular biology and sharpening my analytical mind by asking the right questions at the right time.

Dr. Marc Schneider is thanked for the many hours sweating in front of the CLSM together

Prof. Dr. Ingolf Bernhardt is thanked for giving me the opportunity to perform my radioactive experiments in his „Zentrales Isotopenlabor".

Leon Muijs is thanked for his help with the histological pictures.

Fam. Klopp and Fam. Schradt are thanked for providing me with rabbit eyes.

Furthermore I would like to thank my collegues in our department for helping me in various situations. Special thanks go to Katharina, Samah, Eva, Andreas, Susanne, Petra, Heike, Renate, Nadja, Edeltraud, Nicole and Christiane for giving me a good time.

Hovhannes J. Gukasyan, PhD. Is thanked for supporting me during my stay in the US, especially in the beginning.

Acknowledgements

I thank Jennifer Links for always supporting me morally and keeping up the good spirit the whole time at USC.

I want to thank my parents and family for supporting me financially and morally for so many years and for helping me to carry on in hard times.

Last but not least, my thanks go out to Michel Haas, PhD. For always being there, giving me strength to carry on, being the shoulder to cry on, believing in my skills and ideas, if I didn't. Without you this thesis would not have been possible. Having you by my side is a valuable gift to me!