JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 299–307

Received September 12, 2007
Accepted November 30, 2007
DOI 10.1211/jpp.60.3.0004
ISSN 0022-3573

Cell culture models of the human cornea – a comparative evaluation of their usefulness to determine ocular drug absorption in-vitro

Stephan Reichl

Abstract

Cell culture models of the cornea are continually developed to replace the isolated animal cornea for transcorneal drug absorption studies. The aim of this study was to determine and compare epithelial tightness and permeability of currently available corneal cell culture models to avoid interlaboratory variability and to assess their usefulness for in-vitro permeation studies. Pure epithelial cell culture models (CEPI, SIRC and HCE-T cell lines), primary cultures of human corneal epithelium (HCEpiC) and the two commercially available models (RHC and Epiocular), as well as organotypic human cornea constructs (HCC, HCC-HCE-T), were investigated and data were compared with those obtained from the excised bovine cornea. Barrier properties were assessed by measurements of transepithelial electrical resistance (TEER) and permeability of three passively absorbed substances (mannitol, testosterone and timolol maleate) with different physico-chemical properties. TEER experiments revealed weak barrier functions for all of the investigated epithelial models ($\leq$100–200 $\Omega\,cm^2$), except the HCE-T cell line. Transport studies confirmed TEER results insofar that models showing low TEER values also had higher permeation rates in comparison with the excised bovine cornea. However, models based on HCE-T cells demonstrated similar barrier properties to isolated corneal tissue. The corneal models investigated in our laboratory show clear differences in epithelial barrier function. In-vitro systems comprising the HCE-T cell line seem to be most appropriate to replace excised animal cornea for assessing corneal permeability.

Introduction

In the last decade, the average number of eye diseases requiring treatment has steadily increased. Specifically considering the treatment of glaucoma, the number of prescribed doses in Germany has risen from 382 million DDD (defined daily doses) in 1996 to 432 million DDD in 2005 (Lohse 2006). Eye disease treatments are mostly administered by topical application of ophthalmic drugs, normally to the cul de sac of the eye. Thus, a major focus of drug distribution and penetration is on the conjunctival–scleral pathway and the cornea. Even though the conjunctival–scleral pathway can be highly efficient for intraocular drug delivery, and despite the fact that it bypasses corneal and local vasculature, only a few (high-molecular-weight) substances are delivered using this route. In contrast, the transcorneal route is still the major pathway for ocular drug delivery, representing the direct pathway into the eye. Moreover, it is applicable for most drug substances (Lee & Robinson 1986; Järvinen et al 1995).

Unfortunately, the bioavailability of ophthalmic drugs, whose site of action is intraocular, is rather poor. This is because of the short retention time of drugs at the eye surface due to frequent eye blinking and tear flow, as well as the tight barrier of the corneal epithelium. To overcome these problems, many efforts have been made to increase the retention time of drug formulations at the cornea (Ludwig 2005). Another great challenge in the development of new formulations and excipients is the enhancement of the permeation rate of drugs across epithelial barriers to increase the bioavailable fraction without additional toxic effects (Kaur & Smitha 2002). For these reasons, several studies are being performed

Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Braunschweig, Germany

Stephan Reichl

Correspondence: Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Mendelssohnstr. 1, Braunschweig 38106, Germany. E-mail: S.Reichl@tu-bs.de

Funding: This work was supported by Deutsche Forschungsgemeinschaft, DFG grant Re 2596/1-1.

to develop new ophthalmic drug formulations with mucoadhesive potential, as well as permeation-enhancing effects, so as not to generate a toxic or allergic response. Since the cornea is the main barrier for topically applied drugs, the utility of permeation-enhancing excipients and formulations is evaluated by comparing transcorneal permeation studies. Because of the poor availability of human donor corneas for experimental purposes, these in-vitro studies are largely performed using excised corneal tissues from laboratory animals or killed livestock. However, the use of such tissues has disadvantages. For example, the sacrifice of animals for experimental purposes is deemed inappropriate, animal maintenance is expensive and it requires extensive man-hours. Moreover, procurement of homogeneous materials from slaughtered animals is very difficult and variances of observed data from non-homogeneous animal tissue specimens are unavoidable. Extension of such animal data to a human setting is also a formidable issue.

To overcome the drawbacks and to replace excised corneas from animals, several corneal cell culture models have been established. The intentions driving development of corneal models are very different. These models have been used to study the physiological features of corneal tissues and corneal cells. Moreover, corneal equivalents have been cultivated for wound healing studies (Minami et al 1993; Griffith et al 1999; Germain et al 2000). Since the establishment of the 3R principle (Replace, Reduce, Refine) in 1959 by Russell and Burch, and following the establishment of guidelines that ban animal-based drug tests by the cosmetics industry in the European Union in 2003 (2003/15/EG), immense efforts have been made to improve these cell culture models so that they may serve as a substitute for animal experiments. A variety of corneal epithelial cell culture models have been established in recent years to replace the eye irritation test (Draize test), an animal experiment that is performed on rabbits. In addition to simple models of corneal epithelium, three-dimensional organotypic corneal equivalents have been described for this purpose as well (Kruszewski et al 1997; Schneider et al 1997; Clothier et al 2000; Zorn-Kruppa et al 2005). Meanwhile, two models of corneal epithelium for in-vitro toxicity studies (Epiocular from MatTek and RHC model from SkinEthic) are commercially available. These models were mainly developed to serve as tools for toxicity and eye irritation testing.

Moreover, in recent years, some cell culture models for drug transport and permeation studies based on corneal epithelial cells as well as organotypic cornea constructs have been made available (Hornof et al 2005). For reasons mentioned above, more efforts have been made to develop cell culture models based on human sources in recent years. Thus, some corneal models are used in studies that deal with drug absorption and metabolism; however, up to now, no comparisons of permeation data have been described for the existing models in the same laboratory to avoid interlaboratory variability. Therefore, the aim of this study is to compare corneal epithelium models and three-dimensional cornea equivalents, including the three corneal cell types (endothelial, stromal and epithelial cells), to evaluate their usefulness as in-vitro models for permeation studies. The tightness of epithelial tissues due to the presence of tight junctions was investigated by measuring the transepithelial electrical resistance (TEER). Furthermore, the permeabilities of the cell culture models for mannitol (paracellular marker), testosterone (transcellular marker) and timolol maleate (ophthalmic model drug) were determined and compared with those obtained using excised bovine cornea. Cell culture models included in this investigation were primary cultures of human corneal epithelium (HCEpiC), the frequently used rabbit cell line SIRC (SIRC – Statens Seruminstitut Rabbit Cornea), two different immortalized cell lines of human corneal epithelial cells CEPI and HCE-T, and the commercially available models Epiocular and RHC, as well as organotypic human cornea constructs HCC and HCC-HCE-T.

Material and Methods

Cell culture models and corneal tissues

SIRC

The SIRC cell line is an immortalized cell line established by M. Volkert of the Statens Seruminstitut, Copenhagen, Denmark, from the cornea of a normal rabbit in 1957. It has been used as a model for drug absorption by Goskonda and colleagues (Goskonda & Reddy 1999; Goskonda et al 1999, 2000). Mitra and co-workers have frequently used this cell line in studies of corneal drug transport and drug metabolism during transcorneal passage (Tak et al 2001; Dey et al 2003; Majumdar et al 2003). In contrast to other epithelial cell lines, the SIRC cells do not exhibit an epithelial, cobblestone-like morphology; rather, they have a fibroblast phenotype (Niederkorn et al 1990).

SIRC cells were purchased from European Collection of Cell Cultures (ECACC, UK-Wiltshire; Cat. No. 89090404). Many variations of SIRC growth medium have been described in literature. In this study, the standard ECACC culture medium was used (SIRC 1) and the same medium, including additional supplements, was used as described by Goskonda and colleagues (Goskonda & Reddy 1999; Goskonda et al 1999) (SIRC 2).

CEPI

CEPI cells were a kind gift from Nestec (Lausanne, Switzerland). This cell line was established from human corneal epithelial cultures by means of SV40 transfection (Sharif et al 1998). It is a well characterized cell line (Offord et al 1999). CEPI cells have not been described as a pure corneal epithelial model for drug absorption studies. However, they have been used to build up cornea equivalents, which are intended to replace excised animal corneas in drug permeation experiments (Reichl et al 2004). In this study, CEPI cells were cultured using a standard growth and a keratinocyte growth medium containing a reduced content of fetal calf serum (CEPI, serum-reduced).

HCE-T

HCE-T cells were provided by Dr Araki-Sasaki (Kagoshima, Japan), who established the cell line by infection of human corneal epithelial cells with a recombinant SV40-adenovirus vector in 1995 (Araki-Sasaki et al 1995). HCE-T cells are currently the most frequently investigated

human corneal epithelial cell line. Furthermore, this cell line has been intensively used by Urtti and co-workers and represents a standard tool for drug permeation, bioavailability prescreening and toxicity assessment (Saarinen-Savolainen et al 1998; Toropainen et al 2001, 2003; Ranta et al 2003; Becker et al 2007).

HCEpiC

Isolation and standard cultivation procedure of primary cultures from human corneal epithelium, as well as culture for several population doublings, are considered as difficult and normally require cultivation under serum-free conditions. Meanwhile, some cell cultures are commercially available. HCEpiC cells were purchased from ScienCell (San Diego, CA, USA). These cultures have been described up to now in studies regarding growth characteristics and wound healing (Pappa et al 2005; Yang et al 2005; Zhang et al 2005).

Cultivation of epithelial models

All epithelial models were cultivated in the same way: 100 000 cells/cm^2 of each cell line were seeded onto Transwell inserts (polycarbonate, 1.13 cm^2, 3 μm pore size) and maintained for 7 days under liquid-covered conditions. Thereafter, the cultures were switched to air-interfaced condition and cultivated for an additional 10 days, forming a multilayered corneal epithelium. Medium was changed three times per week. A brief summary of the epithelial models, as well as the used growth media, is listed in Table 1.

Epiocular

Epiocular was purchased from MatTek Corporation (Ashland, MA, USA). This commercially available model was generated to test ocular cytotoxicity and irritancy in-vitro (Jones et al 2001). In contrast to the models described above, the Epiocular model consisted of normal, human-derived epidermal keratinocytes (not of corneal origin) that have been cultured to form a stratified, squamous epithelium with a morphology similar to corneal epithelium. The samples were handled according to the manufacturer's protocol and were used within 24 h of arrival.

Technical data for this model are listed in Table 1.

RHC

Reconstituted human corneal epithelial tissue, also referred to as the RHC model, is also commercially available and was purchased from SkinEthic Laboratories (Nice, France). This model consists of immortalized human corneal epithelial cells (HCE) that are cultivated on 0.5-cm^2 polycarbonate filters at the air–liquid interface for 5 days in chemically defined medium. During this time, a multilayered tissue forms with a morphology and ultrastructure similar to those observed in-vivo. It was created and intensively investigated as a replacement for the Draize rabbit eye irritation test (Doucet et al 2006). Studies using RHC as an in-vitro model for testing transcorneal permeation of drugs have not been described yet. The samples were handled according to the manufacturer's protocol and were used within 24 h of arrival.

Technical data for this model are listed in Table 1.

Table 1 Summary of the technical specifications of the epithelial cell culture models

	Origin	Growth medium
Epiocular	Human-derived epidermal keratinocytes (from neonatal-foreskin tissue)	DMEM, supplemented with insulin, hydrocortisone and other stimulators of nonkeratinizing epithelial differentiation, gentamicin, amphotericin B
RHC	Transformed human corneal epithelial cells, cell line HCE	MCDB 153 chemically defined, supplemented with insulin, hydrocortisone, $CaCl_2$, gentamicin
SIRC 1	Immortalized rabbit corneal epithelial cells, Statens Seruminstitut	MEM, supplemented with 10% FCS, 2 mM glutamine, 1% non-essential amino acids, 1% pen/strep, amphotericin B
SIRC 2	See SIRC 1	as SIRC 1, additionally 1.76 mg mL^{-1} lactalbumin hydrolysate, 0.57 mg mL^{-1} yeast extract
CEPI	SV40 transformed human corneal epithelial cells, cell line CEPI 17 CL 4	DMEM/F12, supplemented with 5% FCS, 2 mM glutamine, 5 μg mL^{-1} insulin, 1.4 μM hydrocortisone, 1 ng mL^{-1} EGF, 10 μg mL^{-1} transferrin, 1% pen/strep, amphotericin B
CEPI sr (serum-reduced)	See CEPI	MCDB 153, supplemented with 25 μg mL^{-1} BPE, 5 μg mL^{-1} insulin, 5 ng mL^{-1} EGF 1.4 μM, hydrocortisone, 0.5% FCS, 10 μg mL^{-1} transferrin, 1% pen/strep, amphotericin B
HCE-T	SV40 transformed human corneal epithelial cells, cell line HCE-T	DMEM/F12, supplemented with 5% FCS, 5 μg mL^{-1} insulin, 10 ng mL^{-1} EGF, 0.5% DMSO, 0.1 μg mL^{-1} cholera-toxin, 1% pen/strep, amphotericin B
HCEpiC	Human corneal epithelial cells, primary cell culture	Keratinocyte medium supplemented with 10 μg mL^{-1} BSA, 5 μg mL^{-1} transferrin, 50 μg mL^{-1} BPE, 2.5 μg mL^{-1} insulin, 5 ng mL^{-1} FGF-2, 500 ng mL^{-1} adrenaline, 0.5 μg mL^{-1} hydrocortisone, 10^{-8} M prostaglandin E_2, 30 nM T_3, 1% pen/strep

HCC and HCC-HCE-T

Besides the pure epithelial models, two different organotypic human cornea constructs (HCC and HCC-HCE-T) were included in the study. Such corneal equivalents include three corneal cell types (epithelial, stromal and endothelial cells), as well as extracellular matrix (normally collagen), and are reconstructed step-by-step by means of tissue engineering techniques (Minami et al 1993). In previous investigations, it could be shown that these constructs exhibit morphology and ultrastructural histology that are similar to the original. In addition, similar results were obtained for both cornea constructs, as well as excised corneas, in transcorneal drug absorption studies (Tegtmeyer et al 2001; Reichl et al 2004).

The HCC and HCC-HCE-T cornea equivalents were constructed as described previously (Reichl et al 2004). Briefly, immortalized cell lines (i.e. SV40 transfected human corneal epithelial (CEPI cells in the case of HCC and HCE-T cells in the case of HCC-HCE-T) and endothelial cells (HENC)), and primary isolated native human corneal fibroblasts were used for reconstruction of the human cornea in-vitro. The standard cultivation and characterization of these cells were reported previously (Bednarz et al 2000). Endothelial cells (2×10^5) were seeded onto a 4.5-cm^2 Transwell insert (polycarbonate filter) covered with a thin layer of type I collagen, acid-extracted from rat tail, and grown to confluence within 7 days in F99 medium, a 1:1 medium mixture of Ham's F12 and Medium 199 (Gibco BRL Life Technologies, Karlsruhe, Germany) supplemented with 5% fetal calf serum (FCS; Biochrom KG, Berlin, Germany) and 1% antibiotic/antimycotic solution (Gibco BRL). A type I collagen gel matrix containing 6×10^4 stromal fibroblasts was then cast atop the confluent endothelial cell layer. An endothelium stroma equivalent was cultivated for approximately 4 days submerged in DMEM supplemented with 10% FCS, 4 mM L-glutamine and 1% antibiotic/antimycotic solution (PAA Laboratories, Linz, Austria). Epithelial cells (1.5×10^5; CEPI and HCE-T, respectively) were seeded onto the contracted collagen lattice and grown in DMEM/F12 medium – a 1:1 medium mixture of DMEM (Gibco BRL) and Ham's F12 (Gibco BRL) supplemented with 5% FCS, 5 μg mL^{-1} insulin, 1.4 mM hydrocortisone, 1 ng mL^{-1} epidermal growth factor (EGF), 10 μg mL^{-1} transferrin (Biochrom), 2 mM L-glutamine and 1% antibiotic/antimycotic solution, in the case of CEPI cells – for an additional 7 days until confluence. The HCE-T growth medium consisted of DMEM/F12 as well supplemented with 5% FCS, 5 μg mL^{-1} insulin, 10 ng mL^{-1} EGF (Biochrom), 0.5% dimethyl sulfoxide, 0.1 μg mL^{-1} cholera toxin (Sigma, Deisenhofen, Germany) and 1% antibiotic/antimycotic solution (Gibco BRL). After the epithelium became confluent, the tissue construct was lifted to the air–liquid interface for an additional 10 days and cultivated in DMEM/F12 medium with a reduced serum content of 2%. Within 10 days, a multilayered epithelium was formed. The cultures were maintained in a humidified incubator at 37°C with 5% CO_2 and medium was replaced three times per week.

Excised bovine cornea

Eyes from 6-month-old cattle were obtained from the slaughterhouse of the Institute for Animal Science and Animal Husbandry (Braunschweig, Germany). The corneas were excised directly after slaughtering with an attached 1–2 mm wide scleral ring, cleaned by washing three times with phosphate-buffered saline, and were used immediately.

Transepithelial electrical resistance

Transepithelial electrical resistance (TEER) was measured using an epithelial voltohmmeter EVOM (World Precision Instruments, Sarasota, USA) and the results were corrected for the background introduced by the blank filter. TEER was used as an indicator of epithelial tightness and functional integrity. Before, and at the end, of each permeability experiment, TEER was measured to determine the condition of the cells. The TEER data did not significantly change during the course of the permeability studies.

Permeation studies

The permeation studies were performed using 1-^{14}C-mannitol and 4-^{14}C-testosterone (specific activity, 50–55 mCi $mmol^{-1}$; Biotrend, Köln, Germany) as hydrophilic and lipophilic markers to characterize paracellular and transcellular permeation, respectively. Aqueous isotonic phosphate-buffered saline, pH 7.4 (PBS), containing 0.5% timolol maleate (0.5% THM) (Ciba-Vision, Weßling, Germany), an ophthalmic model drug, was also used as the donor. The experiments were performed at 37°C and, in the case of epithelial models, they were initiated by adding 3.5 mL serum-free DMEM/F12 medium to the basolateral side (receiver side) and 0.25 mL serum-free medium containing mannitol or testosterone (4 μCi mL^{-1}) and 0.5% THM, respectively, to the apical side (donor side). The receiver solution was stirred with a magnetic stirrer at 100 rev min^{-1} during the experiment. At intervals of 30 min, until 300 min, volumes of 100 μL were withdrawn from the receiver chamber and replaced with an equal volume of blank medium. In the cases of excised corneas and cornea constructs, permeation studies were performed using Franz diffusion cells, as described earlier (Reichl et al 2004). Partition coefficients, P, between 1-octanol and medium or PBS at pH 7.4 were determined using the slow-stirring technique with three different concentrations at 25°C.

Calculation of flux values and permeation coefficients

Radioactivity was measured using a liquid scintillation counter (PW 4700; Philips, Hamburg, Germany) after adding 1.5 mL scintillation liquid (OptiPhase SuperMix; Fisher Chemicals, Loughborough, UK) to every sample. THM samples were analysed by an HPLC method, as described earlier (Reichl et al 2005).

The permeation profiles of donor substances were determined by plotting the amount of drug that permeated through the excised cornea, epithelial model or cornea construct ($\mu g\,cm^{-2}$) versus the time (min). The steady-state flux (J) values across corneal tissues were evaluated from the linear ascents of the permeation graphs by means of the relationship:

$$J = dQ/dtA \ (\mu g\,cm^{-2}\,s^{-1}) \quad (1)$$

where Q is the quantity of substance crossing epithelial models, human cornea constructs or excised cornea; A is the corneal area exposed; and t is the time of exposure. The permeation coefficient, P, was calculated as:

$$P = J/c_o \ (cm\,s^{-1}) \quad (2)$$

whereas c_o represents the initial drug concentration in the donor compartment.

Data analysis

Each experiment was performed in sextuplicate. Statistical data analyses were carried out using analysis of variance, followed by Student–Newman–Keuls post-hoc tests ($P < 0.05$).

Results and Discussion

The cornea is a transparent tissue in the eye that is responsible for the refraction of incoming light. Furthermore, the cornea is an important mechanical and chemical barrier that limits the access of exogenous substances to the eye and protects the intraocular tissues. It is the major absorption route for topically applied drugs, even though it forms a tight barrier. The human cornea is a multilayered tissue made up of three major cell layers: epithelium, stroma and endothelium. Due to the presence of tight junctions (zona occludens), the epithelium is the rate-limiting barrier for the permeation of hydrophilic and moderately lipophilic drugs. The stroma and endothelium offer very little resistance to transcorneal permeation (Klyce & Crosson 1985; Huang et al 1989), although highly lipophilic substances are altered in their diffusion due to the hydrophilic characteristics of the stroma (Huang et al 1983). Therefore, most cell culture models of corneal tissue comprise only epithelial cells (Hornof et al 2005). However, to reflect the in-vivo properties of the cornea more accurately, epithelium-stroma-equivalents (Parnigotto et al 1998; Builles et al 2007), as well as cell culture models of the whole cornea, consisting of epithelial, stromal and endothelial cells, have been developed (Griffith et al 1999; Tegtmeyer et al 2001; Reichl & Müller-Goymann 2003). An ideal corneal cell culture model for in-vitro permeation studies should exhibit a similar multilayered architecture to that observed in-vivo, form a tight epithelium and have barrier properties similar to excised cornea for a wide spectrum of substances and drugs with different physico-chemical characteristics.

All investigated models, including pure epithelial cell cultures, as well as organotypic constructs and commercially available models, were cultivated under air–liquid interface conditions. Such a technique results in multilayered cell growth and promotes cell differentiation and tissue-specific characteristics. In addition, culturing cells under an air interface condition is considered to promote formation of tight corneal epithelial cell layers (Kawazu et al 1998; Chang et al 2000; Toropainen et al 2001). The expression of functional zona occludens is usually characterized by electrophysiological parameters, such as the transepithelial electrical resistance (TEER). The results obtained from corneal epithelial models are presented in Figure 1. For corneal tissues, particularly those from rabbits, TEER values in the range of 1000–3300 (Klyce 1972; Rojanasakul et al 1992; Kikuchi et al 2005) and $7500\,\Omega \cdot cm^2$ (Marshall & Klyce 1983) have been reported. However, none of the pure epithelial models, except HCE-T, exhibited a resistance within this range. The SIRC and CEPI cell lines, as well as the primary culture HCEpiC, had TEER values $\leq 100\,\Omega\,cm^2$; the HCE-T model values were about $1300\,\Omega\,cm^2$. In the case of the RHC model (Skinethic) and Epiocular (Mattek), respective values of 150 and $200\,\Omega\,cm^2$ were observed. Consequently, among the investigated corneal models, only the HCE-T model exhibited epithelial tightness and functional integrity, while the TEER values of the others were far below the TEER values reported for isolated rabbit cornea. To date, the HCE-T cell line is the most thoroughly characterized model of human corneal epithelium. TEER values obtained with HCE-T in the range of 400–800 $\Omega\,cm^2$ and 300–600 $\Omega\,cm^2$ have been reported by Toropainen et al (2001) and Becker et al (2007), respectively. In contrast, the CEPI cell line, which has been used for reconstruction of human cornea (Reichl et al 2005), did not possess TEER values greater than $100\,\Omega\,cm^2$, although a multilayered epithelium was observed. Moreover, reduction of serum content in the growth medium, which has been reported to promote the formation of tight junctions (Eisenblätter et al 2002), did not result in an increase of TEER in the case of CEPI cells (CEPI, serum-reduced). The rabbit cell line SIRC was cultured in two different media. In both cases, the formation of tight epithelial sheets was not observed. This cell line, which is widely used in studies of cytotoxicity, metabolism and permeation of new drug substances (Goskonda & Reddy 1999; Goskonda et al 1999, 2000); Tak et al 2001; Kitagaki et al 2006), was not able to express a zona occludens in our laboratory. Similar results and findings have been described by Tak et al (2001) and Becker (2006), whereas Goskonda and co-workers reported TEER values $\geq 2000\,\Omega\,cm^2$. The HCEpiC model, which is composed of primary cultures of

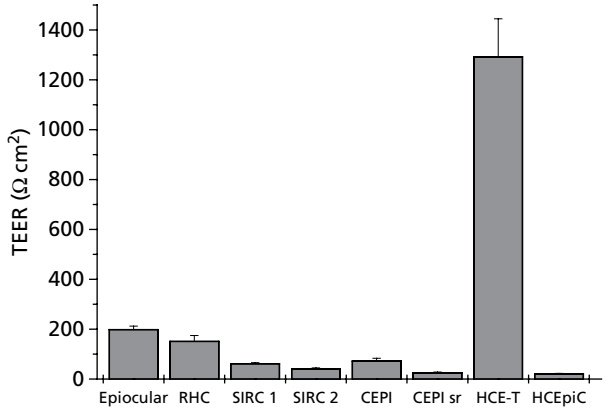

Figure 1 Transepithelial electrical resistance (TEER) of the epithelial models. Each point represents the mean ± s.d. of 6 independent determinations.

human corneal epithelial cells and is cultured in serum-free medium, did not show TEER values more than $100\,\Omega\,cm^2$, suggesting that the cell layers have weak barrier properties. The commercially available RHC model is based on transformed human corneal epithelial cells, as is the HCE-T model, and it shows a well-organized, multilayered epithelial structure. Unfortunately, a TEER value of $150\,\Omega\,cm^2$ was found for RHC, which is much lower than that observed for HCE-T and excised tissue. Even though the Epiocular model showed a slightly higher TEER than RHC, the TEER value of $200\,\Omega\,cm^2$ measured for this model is not considered to be high enough either. An additional drawback of Epiocular is the fact that it is built up by epidermal keratinocytes and not corneal cells.

To further evaluate the different models and their usefulness as in-vitro permeation models, respectively, permeation studies were performed using highly hydrophilic mannitol (Log $P_{oct/med} = -2.0$; where P is the partition coefficient between 1-octanol and the medium or PBS; pH 7.4) as a paracellular marker and highly lipophilic testosterone (Log $P_{oct/med} = 1.9$) as a transcellular marker. As an ophthalmic model drug of medium polarity, timolol maleate (Log $P_{oct/PBS} = -0.2$) was investigated. The results, which are expressed as permeation coefficients, are presented in Table 2 and can be visualized in Figure 2A–C. The permeation results obtained for mannitol confirm the findings of the TEER measurements. Only the HCE-T model and HCC-HCE-T, the organotypic construct comprising the same HCE-T cell line, exhibited permeabilites in the range of $10^{-7}\,cm\,s^{-1}$, as was observed for the excised bovine cornea and was previously reported for isolated corneal tissue of different species (Toropainen et al 2001; Kompella et al 2006). In the case of the other epithelial models, including Epiocular and RHC, as well as the cornea construct HCC, mannitol permeability was increased by an order of magnitude relative to HCE-T, HCC-HCE-T and the excised bovine cornea. Based upon the TEER values and permeation coefficients for mannitol, only the HCE-T model and the cornea construct including this cell line (HCC-HCE-T), among all investigated cell culture models, were found to form tight epithelial sheets similar to those present in the cornea in-vivo.

In the case of excised bovine cornea, a ten-fold increase in the permeability for testosterone was detected relative to mannitol. The organotypic cornea equivalent HCC showed a similar permeation coefficient to that of the isolated cornea, while the HCC-HCE-T and HCE-T model exhibited a two- to four-fold increased permeability. Every epithelial model, including the commercially available models, revealed permeation coefficients that were increased by about ten fold relative to the isolated cornea. These facts lead to the conclusion that, for lipophilic testosterone, the stroma tissue of the cornea, which is also part of the artificial cornea constructs, provides a considerable barrier. For the ophthalmic model drug timolol maleate, the two models based on HCE-T have similar permeation rates to the bovine cornea, although the values tended to be higher by a factor of three. All of the other epithelial models showed increased values up to one order of magnitude and more. The permeation coefficient values of the RHC, Epiocular and HCC models were between these values.

Among the corneal cell culture models investigated in our laboratory, only the models based on the HCE-T cell line appear to be appropriate substitutes for the excised cornea in transcorneal absorption studies. For the purpose of screening studies to investigate the influence of excipients, preservatives, etc., on tight junction function, the pure epithelial HCE-T model seems to be the best choice because of its high TEER and barrier tightness to paracellular marker substances. In addition, it is noticeably easier to cultivate and validate than is the whole organotypic construct. However, for investigations of mechanistic transcorneal transport of new drug substances or bioequivalence, the HCC-HCE-T model seems to be more advantageous since it comprises a tight epithelium and a stroma equivalent, which is of concern in the case of highly lipophilic drugs. The two commercially available models do not appear to be useful as in-vitro permeation models because

Table 2 Drug permeability of the different corneal cell culture models compared with excised bovine cornea

	Permeation coefficient ($10^{-6}\,cm\,s^{-1}$)		
	Mannitol	Testosterone	Timolol maleate
Epiocular	3.18 ± 0.23	26.5 ± 1.0	2.38 ± 0.18
RHC	6.24 ± 0.18	38.4 ± 2.9	6.33 ± 0.09
SIRC 1	8.67 ± 1.27	35.5 ± 2.8	9.83 ± 0.90
SIRC 2	11.2 ± 0.9	26.2 ± 2.9	10.9 ± 0.3
CEPI	9.36 ± 0.65	25.6 ± 2.4	10.4 ± 0.28
CEPI sr	17.3 ± 0.23	28.5 ± 0.23	16.7 ± 0.23
HCEpiC	19.0 ± 1.8	31.7 ± 3.7	13.6 ± 1.7
HCE-T	0.56 ± 0.04	11.4 ± 0.28	3.95 ± 0.51
HCC	5.62 ± 0.90	4.33 ± 0.84	8.4 ± 0.83
HCC-HCE-T	0.92 ± 0.10	6.32 ± 0.55	3.77 ± 0.42
Bovine Cornea	0.26 ± 0.04	2.74 ± 0.33	1.06 ± 0.19

Data are expressed as mean ± s.d., n = 6.

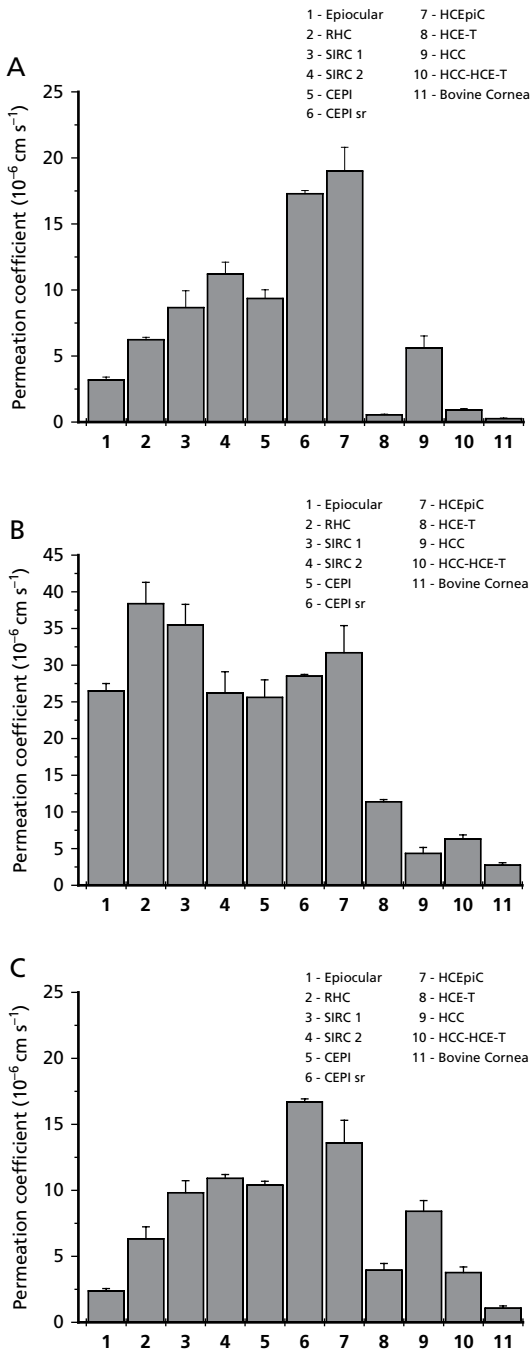

Figure 2 Drug permeability of the different corneal cell culture models compared with excised bovine cornea. Permeation coefficients, P, of mannitol (A), testosterone (B) and timolol maleate (C) are shown. Each point represents the mean ± s.d. of 6 independent determinations.

the epithelium they form is not tight enough. Moreover, they do not exhibit an adequate limitation of lipophilic drug transport. In contrast, their usefulness as in-vitro models for cytotoxicity studies and replacement of animal testing has been reported several times (van Goethem et al 2006). Neither CEPI cells nor SIRC cells show sufficiently high TEER values, even with variable growth media compositions. The permeation coefficients of these cell culture models in comparison with isolated cornea were too high as well. Even though the use of SIRC cells has been reported as an alternative to excised cornea for transcorneal absorption studies (Goskonda et al 1999a, b, 2000), this model was not shown to be useful for in-vitro permeation studies in our laboratory. This is in accordance with data from a previous report (Becker 2006). Furthermore, commercially available primary cultures of human corneal epithelium HCEpiC are not an appropriate in-vitro permeation model as they are not able to generate tight tissues under the chosen growth conditions. However, to date, a useful in-vitro permeation model based on human primary cell cultures has not been described (Hornof et al 2005).

Besides the investigated cornea models, additional cell culture models are commercially available, although they are not widely used at this time. The ECACC distributes a transfected rabbit corneal epithelial cell line (RCE) that has been used for in-vitro permeation studies (Burgalassi et al 2004; Di Colo et al 2004). However, this RCE cell line also shows TEER values of only about 150 $\Omega\,cm^2$, so its usefulness as an in-vitro permeation model remains doubtful. Furthermore, there is a new corneal epithelial model available from Lonza (B-Verviers), which is based upon transfected human corneal epithelial cells. However, to date, published data showing its usefulness as an in-vitro permeation model are not available. Its TEER values of about 800 $\Omega\,cm^2$, described by Becker (2006), suggest that this model forms a tight barrier and could be used for testing transcorneal drug absorption.

However, during recent years more and more studies have shown that numerous compounds are also absorbed by active transport processes (Mannermaa et al 2006). Furthermore, the expression of efflux transport systems located in corneal epithelium has been reported for rabbit and human tissue (Kawazu et al 1999; Dey et al 2003; Karla et al 2007a, b; Becker et al 2007). These ABC transporters seem to play a vital role in transcorneal penetration for certain compounds (Dey et al 2004). Thus, a next step towards an optimal corneal in-vitro model has to be the evaluation of the different models regarding their expression of active transport systems in culture.

Conclusion

The adequate replacement of animal testing for investigation of the transcorneal absorption behaviour of passively absorbed drugs could only be shown for corneal models (pure epithelium as well as a cornea construct) based on the HCE-T cell line in this study. Use of the HCE-T cell culture model seems to be optimal for studies of epithelial barrier properties (tight junctions). On the other hand, the complete human cornea construct might be able to serve as a substitute for isolated animal cornea during the development phase of new ophthalmic drugs or for bioequivalence studies of pharmaceutical preparations. The HCC-HCE-T model is currently under investigation (pre-validation phase) as an in-vitro model for pharmacokinetic studies conducted in the course of the authorization procedure for marketing. However, further studies are required to elucidate the expression pattern of transport proteins in corneal in-vitro models.

References

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

Becker, U. (2006) *Examination of the penetration behavior of pharmaceutical drugs on the eye and set-up of an in vitro cell model for ocular drug delivery*. PhD thesis, Saarland University, Saarbrücken. http://scidok.sulb.uni-saarland.de/volltexte/2007/946/index.html (accessed 03 July 2007)

Becker, U., Ehrhardt, C., Daum, N., Baldes, C., Schaefer, U. F., Ruprecht, K. W., Kim, K. J., Lehr, C. M. (2007) Expression of ABC-transporters in human corneal tissue and the transformed cell line, HCE-T. *J. Ocul. Pharmacol. Ther*. **23**: 172–181

Bednarz, J., Teifel, M., Friedl, P., Engelmann, K. (2000) Immortalization of human corneal endothelial cells using electroporation protocol optimized for human corneal endothelial and human retinal pigment epithelial cells. *Acta Ophthalmol. Scand*. **78**: 130–136

Builles, N., Bechetoille, N., Justin, V., André, V., Barbaro, V., Iorio, E. Di., Auxenfans, C., Hulmes, D. J. S., Damour, O. (2007) Development of a hemicornea from human primary cell cultures for pharmacotoxicology testing. *Cell Biol. Toxicol*. **23**: 279–292

Burgalassi, S., Monti, D., Brignoccoli, A., Fabiani, O., Lenzi, C., Pirone, A., Chetoni, P. (2004) Development of cultured rabbit corneal epithelium for drug permeation studies: a comparison with excised rabbit cornea. *J. Ocul. Pharmacol. Ther*. **20**: 518–532

Chang, J. E., Basu, S. K., Lee, V. H. (2000) Air-interface condition promotes the formation of tight corneal epithelial cell layers for drug transport studies. *Pharm. Res*. **17**: 670–676

Clothier, R., Orme, A., Walker, T. L., Ward, S. L., Kruszewski, F. H., DiPasquale, L. C., Broadhead, C. L. (2000) A comparison of three cytotoxicity endpoints in the corneal HCE-T model. *ATLA Alternatives to Laboratory Animals* **28**: 293–302

Dey, S., Patel, J., Anand, B. S., Jain-Vakkalagadda, B., Kaliki, P., Pal, D., Ganapathy, V., Mitra, A. K. (2003) 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

Dey, S., Gunda, S., Mitra, A. K. (2004) Pharmacokinetics of erythromycin in rabbit corneas after single-dose infusion: role of P-glycoprotein as a barrier to in vivo ocular drug absorption. *J. Pharmacol. Exp. Ther*. **311**: 246–255

Di Colo, G., Burgalassi, S., Zambito, Y., Monti, D., Chetoni, P. (2004) Effects of different N-trimethyl chitosans on in vitro/in vivo ofloxacin transcorneal permeation. *J. Pharm. Sci*. **93**: 2851–2862

Doucet, O., Lanvin, M., Thillou, C., Linossier, C., Pupat, C., Merlin, B., Zastrow, L. (2006) Reconstituted human corneal epithelium: a new alternative to the Draize eye test for the assessment of the eye irritation potential of chemicals and cosmetic products. *Toxicol. In Vitro* **20**: 499–512

Eisenblätter, T., Psathaki, K., Nitz, T., Galla, H. J., Wegener, J. (2002) Cell culture media: selection and standardization. In: Lehr, C. M. (ed.) *Cell culture models of biological barriers'* 1st edn. Taylor & Francis, London, pp 20–40

Germain, L., Carrier, P., Auger, F. A., Salesse, C., Guerin, S. L. (2000) Can we produce a human corneal equivalent by tissue engineering? *Prog. Retin. Eye Res*. **19**: 497–527

Goskonda, V. R., Reddy, I. K. (1999) Permeability of pure enantiomers of timolol across SIRC rabbit corneal cells. *Pharm. Pharmacol. Commun*. **5**: 111–115

Goskonda, V. R., Khan, M. A., Hutak, C. M., Reddy, I. K. (1999) 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

Goskonda, V. R., Hill, R. A., Khan, M. A., Reddy, I. K. (2000) Permeability of chemical delivery systems across rabbit corneal (SIRC) cell line and isolated corneas: a comparative study. *Pharm. Dev. Technol*. **5**: 409–416

Griffith, M., Osborne, R., Munger, R., Xiong, X., Doillon, C. J., Laycock, N. L., Hakim, M., Song, Y., Watsky, M. A. (1999) Functional human corneal equivalents constructed from cell lines. *Science* **286**: 2169–2172

Hornof, M., Toropainen, E., Urtti, A. (2005) Cell culture models of the ocular barriers. *Eur. J. Pharm. Biopharm*. **60**: 207–225

Huang, H. S., Schoenwald, R. D., Lach, J. L. (1983) Corneal penetration behavior of beta-blocking agents II: assessment of barrier contributions. *J. Pharm. Sci*. **72**: 1272–1279

Huang, A. J., Tseng, S. C., Kenyon, K. R. (1989) Paracellular permeability of corneal and conjunctival epithelia. *Invest. Ophthalmol. Vis. Sci*. **30**: 684–689

Järvinen, K., Järvinen, T., Urtti, A. (1995) Ocular absorption following topical delivery. *Adv. Drug Deliv. Rev*. **16**: 3–19

Jones, P. A., Budynsky, E., Cooper, K. J., Decker, D., Griffiths, H. A., Fentem, J. H. (2001) Comparative evaluation of five in vitro tests for assessing the eye irritation potential of hair-care products. *Altern. Lab. Anim*. **29**: 669–692

Karla, P. K., Pal, D., Quinn, T., Mitra, A. K. (2007a) Molecular evidence and functional expression of a novel drug efflux pump (ABCC2) in human corneal epithelium and rabbit cornea and its role in ocular drug efflux. *Int. J. Pharm*. **336**: 12–21

Karla, P. K., Pal, D., Mitra, A. K. (2007b) Molecular evidence and functional expression of multidrug resistance associated protein (MRP) in rabbit corneal epithelial cells. *Exp. Eye Res*. **84**: 53–60

Kaur, I. P., Smitha, R. (2002) Penetration enhancers and ocular bioadhesives: Two new avenues for ophthalmic drug delivery. *Drug Dev. Ind. Pharm*. **28**: 353–369

Kawazu, K., Shiono, H., Tanioka, H., Ota, A., Ikuse, T., Takashina, H., Kawashima, Y. (1998) Beta adrenergic antagonist permeation across cultured rabbit corneal epithelial cells grown on permeable supports. *Curr. Eye Res*. **17**: 125–131

Kawazu, K., Yamada, K., Nakamura, M., Ota, A. (1999) 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

Kikuchi, T., Suzuki, M., Kusia, A., Iseki, K., Sasaki, H. (2005) Synergistic effect of EDTA and boric acid on corneal penetration of CS-088. *Int. J. Pharm*. **290**: 83–89

Kitagaki, M., Wakuri, S., Hirota, M., Tanaka, N., Itagaki, H. (2006) Sirc-cvs cytotoxicity test: an alternative for predicting rodent acute systemic toxicity. *J. Toxicol. Sci*. **31**: 371–379

Klyce, S. D. (1972) Electrical profiles in the corneal epithelium. *J. Physiol*. **226**: 407–429

Klyce, S. D., Crosson, C. E. (1985) Transport processes across the rabbit corneal epithelium: a review. *Curr. Eye Res*. **4**: 323–331

Kompella, U. B., Sundaram, S., Raghava, S., Escobar, E. R. (2006) Luteinizing hormone-releasing hormone agonist and transferrin functionalizations enhance nanoparticle delivery in a novel bovine ex vivo eye model. *Mol. Vis*. **12**: 1185–1198

Kruszewski, F. H., Walker, T. L., DiPasquale, L. C. (1997) Evaluation of a human corneal epithelial cell line as an in vitro model for assessing ocular irritation. *Fundam. Appl. Toxicol*. **36**: 130–140

Lee, V. H., Robinson, J. R. (1986) Topical ocular drug delivery: recent developments and future challenges. *J. Ocul. Phamacol*. **2**: 67–108

Lohse, M. J. (2006) Ophthalmika. In: Paffrath, D., Schwabe, U. (eds) *Arzneiverordnungs-Report 2006*. Springer, Berlin, pp 783–806

Ludwig, A. (2005) The use of mucoadhesive polymers in ocular drug delivery. *Adv. Drug Deliv. Rev*. **57**: 1595–1639

Majumdar, S., Tirucherai, G. S., Pal, D., Mitra, A. K. (2003) Functional differences in nucleoside and nucleobase transporters expressed on the rabbit corneal epithelial cell line (SIRC) and isolated rabbit cornea. *AAPS PharmSci*. **5**: E15

Mannermaa, E., Vellonen, K.S., Urtti, A. (2006) Drug transport in corneal epithelium and blood-retina barrier: emerging role of transporters in ocular pharmacokinetics. *Adv. Drug Deliv. Rev*. **58**: 1136–1163

Marshall, W. S., Klyce S. D. (1983) Cellular and paracellular pathway resistances in the tight Cl-secreting epithelium of rabbit cornea. *J. Membr. Biol*. **73**: 275–282

Minami, Y., Sugihara, H., Oono, S. (1993) Reconstruction of cornea in three-dimensional collagen gel matrix culture. *Invest. Ophthalmol. Vis. Sci*. **34**: 2316–2324

Niederkorn, J. Y., Meyer, D. R., Ubelaker, J. E., Martin, J. H. (1990) Ultrastructural and immunhistological characterization of the SIRC corneal cell line. *In Vitro Cell. Dev. Biol*. **26**: 923–930

Offord, E. A., Sharif, N. A., Mace, K., Tromvoukis, Y., Spillare, E. A., Avanti, O., Howe, W. E., Pfeifer, A. M. (1999) Immortalized human corneal epithelial cells for ocular toxicity and inflammation studies. *Invest. Ophthalmol. Vis. Sci*. **40**: 1091–1101

Pappa, A., Brown, D., Koutalos, Y., Degregori, J., White, C., Vasiliou, V. (2005) Human aldehyde dehydrogenase 3A1 inhibits proliferation and promotes survival of human corneal epithelial cells. *J. Biol. Chem*. **280**: 27998–28006

Parnigotto, P. P., Bassani, V., Montesi, F., Conconi, M. T. (1998) Bovine corneal stroma and epithelium reconstructed in vitro: characterisation and response to surfactants. *Eye* **12**: 304–310

Ranta, V. P., Laavola, M., Toropainen, E., Vellonen, K. S., Talvitie, A., Urtti, A. (2003) Ocular pharmacokinetic modeling using corneal absorption and desorption rates from in vitro permeation experiments with cultured corneal epithelial cells. *Pharm. Res*. **20**: 1409–1416

Reichl, S., Müller-Goymann, C. C. (2003) The use of a porcine organotypic cornea construct for permeation studies from formulations containing befunolol hydrochloride. *Int. J. Pharm*. **250**: 191–201

Reichl, S., Bednarz, J., Müller-Goymann, C. C. (2004) Human corneal equivalent as cell culture model for in vitro drug permeation studies. *Br. J. Ophthalmol*. **88**: 560–565

Reichl, S., Döhring, S., Bednarz, J., Müller-Goymann, C. C. (2005) Human Cornea Construct HCC - an alternative for in-vitro permeation studies? A comparison with human donor corneas. *Eur. J. Pharm. Biopharm*. **60**: 305–308

Rojanasakul, Y., Wang, L. Y., Bhat, M., Glover, D. D., Malanga, C. J., Ma, J. K. (1992) The transport barrier of epithelia: a comparative study on membrane permeability and charge selectivity in the rabbit. *Pharm. Res*. **9**: 1029–1034

Russell, W. M., Burch, R. L. (1959) *The principles of humane experimental technique*. Charles Thomas Publishers, online issue available at: http://altweb.jhsph.edu/publications/humane_exp/het-toc.htm (accessed 03 July 2007)

Saarinen-Savolainen, P., Järvinen, T., Araki-Sasaki, K., Watanabe, H., Urtti, A. (1998) Evaluation of cytotoxicity of various ophthalmic drugs, eye drop excipients and cyclodextrins in an immortalized human corneal epithelial cell line. *Pharm. Res*. **15**: 1275–1280

Schneider, A. I., Maier-Reif, K., Graeve, T. (1997) The use of an in vitro cornea for predicting ocular toxicity. *In vitro toxicol*. **10**: 309–318

Sharif, N. A., Wiernas, T. K., Howe, W. E., Griffin, B. W., Offord, E. A., Pfeifer, A. M. (1998) Human corneal epithelial cell functional responses to inflammatory agents and their antagonists. *Invest. Ophthalmol. Vis. Sci*. **39**: 2562–2571

Tak, R. V., Pal, D., Gao, H., Dey, S., Mitra, A. K. (2001) Transport of acyclovir ester prodrugs through rabbit cornea and SIRC-rabbit corneal epithelial cell line. *J. Pharm. Sci*. **90**: 1505–1515

Tegtmeyer, S., Papantoniou, I., Müller-Goymann, C. C. (2001) Reconstruction of an in vitro cornea and its use for drug permeation studies from different formulations containing pilocarpine hydrochloride. *Eur. J. Pharm. Biopharm*. **51**: 119–125

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

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

van Goethem, F., Adriaens, E., Alépée, N., Straube, F., De Wever, B., Cappadoro, M., Catoire, S., Hansen, E., Wolf, A., Vanparys, P. (2006) Prevalidation of a new in vitro reconstituted human cornea model to assess the eye irritating potential of chemicals. *Toxicol. In Vitro* **20**: 1–17

Yang, H., Mergler, S., Sun, X., Wang, Z., Lu, L., Bonanno, J. A., Pleyer, U., Reinach, P. S. (2005) TRPC4 knockdown suppresses EGF-induced store operated channel activation and growth in human corneal epithelial cells. *J. Biol. Chem*. **280**: 32230–32237

Zhang, J., Wu, X. Y., Yu, F. S. (2005) Inflammatory response of corneal epithelial cells to pseudomonas aeruginosa infection. *Curr. Eye Res*. **30**: 527–534

Zorn-Kruppa, M., Tykhonova, S., Beige, G., Bednarz, J., Diehl, H. A., Engelke, M. (2005) A human corneal equivalent constructed from SV40-immortalised corneal cell lines. *ATLA Alternatives to Laboratory Animals* **33**: 37–45