# Evaluation of Cytotoxicity of Various Ophthalmic Drugs, Eye Drop Excipients and Cyclodextrins in an Immortalized Human Corneal Epithelial Cell Line

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*Received January 20, 1998; accepted May 9, 1998*

***Purpose.*** An immortalized human corneal epithelial cell line (HCE) was tested as a screening tool for prediction of topical ocular irritation/toxicity by pharmaceuticals.

***Methods.*** Effects of various drugs, excipients and cyclodextrins (CDs) on viability of HCE cells were evaluated using two *in vitro* cytotoxicity tests, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay and propidium iodide assay.

***Results.*** Mitochondrion-based MTT test was a more sensitive indicator of cytotoxicity than the plasma membrane-based propidium iodide test. The tests revealed following cytotoxic rankings for ophthalmic drugs: dipivefrin > timolol > pilocarpine ≈ dexamethasone; for excipients: benzalkonium chloride (BAC) > sodium edetate ($NA_2$ EDTA) > polyvinyl alcohol (PVA) > methylparaben; and for CDs: α-CD > dimethyl-β-cyclodextrin (DM-β-CD) > sulfobutyl ether β-cyclodextrin ($(SBE)_{7m}$-β-CD) ≈ hydroxypropyl-β-cyclodextrin (HP-β-CD) > γ-CD. In consideration of the *in vivo* clinical situation, the short exposure time (5 min) is more relevant even though toxic effects of some test substances were seen only after longer exposure times (30 and 60 min).

***Conclusions.*** Immortalized HCE cells are a promising tool for rapid cytotoxicity assays of ocular medications. The cell line is potentially useful in predicting the *in vivo* corneal toxicity of ocularly applied compounds.

**KEY WORDS:** cell culture; corneal epithelium; cyclodextrins; cytotoxicity; immortalized cell line; ocular irritation; ophthalmic drugs.

## INTRODUCTION

Significant efforts have been directed toward developing *in vitro* alternatives to replace the animals or to reduce their number in ocular safety assessment. The eye irritation tests in rabbits (i.e. Draize test) are the standard procedures for evaluating topical ocular safety (1). Draize test has been widely criticized for ethical reasons, as well as for the questionable accuracy in predicting the human eye response. An alternative is to use isolated target organs (e.g. the enucleated rabbit or chicken eye, the isolated cornea) for toxicology studies (2,3). In this case, however, animals are needed as a source of the tissues, and the viability of the isolated tissues is questionable. Recently, *in vivo* confocal microscopy has been shown to be useful in characterization of the rabbit corneal changes occurring after ocular application of irritating substances (4).

The progress in research utilizing cell-based *in vitro* methods in safety evaluations has been rapid. Many different cell culture systems and endpoints have been proposed for cytotoxicity screening of drugs, surfactants, solvents and various chemicals (5). Primary corneal epithelial cells can be used for rapid screening of acute topical ocular toxicities of large number of compounds (6–8). The disadvantage of primary cultures is their restricted life span, which necessitates seeding of the new primary cells from animals or human eyes frequently. Immortalized cell line can be grown continuously and, therefore, it should be more practical for screening than primary cells.

In the present work, we evaluated the suitability of recently developed immortalized human corneal epithelial cell line (9) for predicting eye irritation/toxicity potential of some commonly used ophthalmic drugs and pharmaceutical excipients.

## MATERIALS AND METHODS

### Materials

Dipivefrin hydrochloride, pilocarpine hydrochloride, timolol maleate and dexamethasone sodium phosphate were obtained from Leiras Pharmaceuticals (Tampere, Finland). Benzalkonium chloride was purchased from Fluka Chemie AG (Buchs, Switzerland), poly(vinyl alcohol) (PVA, molecular weight = 124 000–186 000 g $mol^{-1}$) from Aldrich Chemicals Company, Inc. (Milwaukee, USA), disodium ethylenediaminetetraacetate ($Na_2EDTA$) from Merck (Darmstadt, Germany) and methyl p-hydroxybenzoate from University Pharmacy (Helsinki, Finland). HP-β-CD (Encapsin®, average DS = 0.6; average MW = 1297.4) was purchased from Janssen Biotech (Olen, Belgium) and DM-β-CD (average DS = 14; average MW 1331.5) from Cyclolab R&D Laboratory Ltd. (Budapest, Hungary). $(SBE)_{7m}$-β-CD (average DS = 7; average MW = 2160) was kindly supplied by CyDex, Inc. (Kansas City, KS). α-, β- and γ-CD were from Wacker-Chemie GmbH (Munich, Germany).

Propidium iodide was obtained from Molecular Probes (Eugene, OR), digitonin from Aldrich Chemical Company, Inc. (Milwaukee, USA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma Chemicals Co. (St. Louis, MO), N,N-dimethyl formamide (DMF) from Fluka and sodium dodecylsulfate (SDS) from Merck. Dulbecco's modified Eagles medium (DMEM) with Ham's nutrient mixture F12 (1:1), L-glutamine, fetal bovine serum (FBS), antibiotic/antimycotic solution, Hanks' balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco (Paisley, United Kingdom). Epidermal growth factor (EGF) and insulin were from Sigma Chemicals and dimethylsulfoxide (DMSO) from Merck. Cholera toxin was purchased from Calbiochem (La Jolla, CA). All other chemicals were analytical grade and were used as received.

### Cell Culture

The immortalization of the human corneal epithelial cells (HCE) has been described earlier (9). Mycoplasma free cells

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of passages 42–50 were used. The cells of the HCE cell line were grown at 37°C in a humidified atmosphere of air with 5% $CO_2$ in a standard medium consisting of DMEM/F12 (1:1), 15% heat-inactivated FBS, 0.3 mg/ml L-glutamine, 5 μg/ml insulin, 0.1 μg/ml cholera toxin, 10 ng/ml EGF, 0.5% DMSO, and 0.1 mg/ml streptomycin, 100 IU/ml benzylpenicillin and 0.25 μg/ml amphotericin B. The cells were plated at $2 \times 10^4$ cells/100 μl/well in Nunc 96-well plates (Roskilde, Denmark). Cells were used for cytotoxicity assays when they became confluent on day 3. Cytotoxicity data were obtained from two different experiments by testing three to five concentrations with four wells per concentration.

## Methods

### *Preparation of Test Solutions*

The following compounds were tested for their cytotoxicity to human corneal epithelial cells: pilocarpine, timolol, dipivefrin, dexamethasone, benzalkonium chloride, EDTA, PVA, methylparaben, α-, β-, γ-, HP-β-CD, DM-β-CD and $(SBE)_{7m}$-β-CD. β-CD was studied at a low concentration (8 mM) due to its poor aqueous solubility. Test substances were dissolved in Hank's balanced salt solution (HBSS) and pH was adjusted to 7.4. Dilution of the test substances in the experiment to the half was taken into account when preparing test solutions. Osmolalities were measured with an Osmostat OM-6020 osmometer (Daiichi Kagaku, Kyoto, Japan). The highest concentrations of pilocarpine, HP-β-CD, DM-β-CD and $(SBE)_{7m}$-β-CD were prepared in water. The solutions of HP-β-CD and DM-β-CD (200 mM) were slightly hypertonic (316 and 372 mOsm $kg^{-1}$), but some solutions of $(SBE)_{7m}$-β-CD (100 and 200 mM) and pilocarpine (384 mM, 8%) were strongly hypertonic (>500 mOsm $kg^{-1}$). Hypertonic control solutions were prepared by adding mannitol to 0.9% sodium chloride solution.

### *MTT Assay*

The MTT assay is a colorimetric method for determining cell viability based on reduction of the yellow tetrazolium salt MTT to a purple formazan dye by mitochondrial succinate dehydrogenase in viable cells. The test was performed according to the procedure of Hansen *et al.* (10). Cells were incubated with the test solutions (100 μl) for 5, 30, or 60 min. After the exposure period, the reaction medium was removed, and 100 μl of fresh DMEM/F12 medium (without serum) and 25 μl of MTT solution (5 mg/ml in PBS) were added to each well and incubated at 37°C for 2 hours. Formazan was solubilized by adding 100 μl of extraction buffer (20% w/v SDS in DMF/water 50:50, pH 4.7) and incubated overnight at 37°C. The optical densities were measured at 570 nm using a Multiscan Plus multiwell scanning spectrophotometer (Labsystems Oy, Helsinki, Finland). $IC_{50}$ values (mM), the concentrations of test substances that reduce MTT metabolism to 50% of control levels were determined using nonlinear curve fitting.

### *Propidium Iodide Assay*

Loss of membrane integrity is often a sign of reduced cell viability or direct action of the chemical on plasma membrane. Nonviable cells may be recognized by their uptake of DNA-binding dyes such as propidium iodide. This dye enters the cells only when their plasma membranes have partly lost their integrity. In cells, propidium iodide binds to DNA and becomes fluorescent.

Propidium iodide assay was modified from that of Nieminen *et al.* (11). Cells were seeded at a density of $2 \times 10^4$ cells/well and cultivated on 96-well microtiter plates as described in the MTT assay. Fluorescence measurements were performed in a Bio-Tek® FL500 fluorescence plate reader (Bio-Tek® Instruments, Inc. Vermont, USA) using 530 nm excitation and 590 emission filters. Briefly, medium was removed from each well and replaced with HBSS containing propidium iodide (50 μM). After incubation of 20 min at room temperature, 100 μl of the test substances were added. Fluorescence values (F) were measured from the plate after 5, 15, 30, and 60 min incubation at room temperature. At the end of the experiment, digitonin (95 μM) was added to the wells and the plate was shaken at room temperature for 20 min. Fluorescence was measured again to obtain the value of 100% cell death ($F_{max}$). Percentage viability was defined as follows:

$$\% \text{ Viability} = 100 - \left[\frac{[F - \text{blank1}]}{[F_{max} - \text{blank2}]} \times 100\right]$$

where F is fluorescence at any given time, $F_{max}$ is fluorescence after the addition of digitonin, blank1 and blank2 are fluorescence values from the wells lacking cells before and after the addition of digitonin. Two independent experiments were performed using four wells for each concentration of test substances.

## RESULTS

### *MTT Assay*

After exposure of 5 min, the dipivefrin was clearly cytotoxic at concentrations of 7 mM (0.25%) or more, while timolol and pilocarpine showed mild cytotoxicity at 63 mM (2%) and 192 mM (4%), respectively. Dexamethasone was not toxic at any tested concentrations (1.3–25 mM). At typical concentrations of eye drops the drugs did not cause significant cellular toxicities (Fig. 1). Cell viability decreased below 50% after

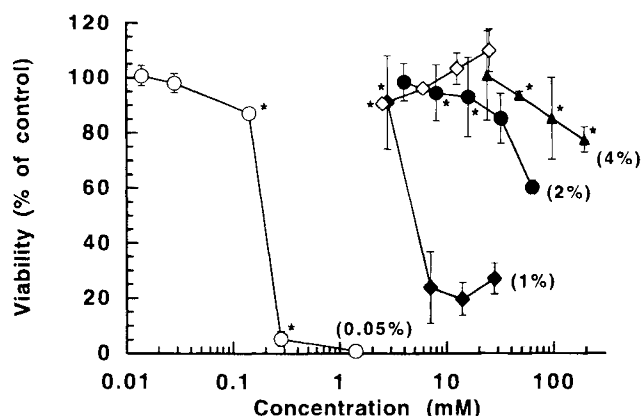


**Fig. 1.** The mitochondrial reduction of MTT in HCE cells after 5 min exposure to BAC (○), dipivefrin (◆), timolol (●), pilocarpine (▲) and dexamethasone (◇) (mean ±SD, n = 8). Typical concentrations of drugs in eye drop preparations have been marked with *.

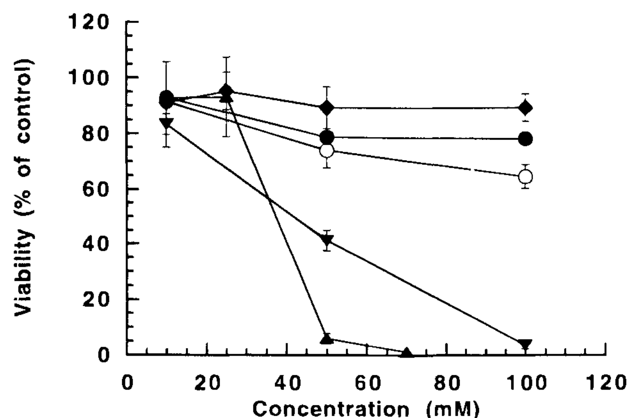


**Fig. 2.** Cytotoxicity of α-CD (▲), γ-CD (●), DM-β-CD (▼), HP-β-CD (◆) and $(SBE)_{7m}$-β-CD (○) to HCE cells in the MTT assay after 5 min exposure time (mean ±SD, n = 8). 100 mM $(SBE)_{7m}$-β-CD solution was strongly hypertonic.

1 h exposure to dipivefrin, timolol and pilocarpine at 2.8 mM (0.1%), 32 mM (1%), and 96 mM (2%), respectively.

The commercial eye drops may contain several pharmaceutical excipients such as PVA, BAC, methylparaben, and EDTA. Cell damage caused by BAC was rapid. In 5 min, all cells were killed using 0.28 mM (0.01%) or more concentrated solutions (Fig. 1). At concentrations of 0.14 mM (0.005%) and less BAC showed only mild cell toxicity at 5 min exposure, but at longer exposures these low concentrations showed significant toxicity (50–80% cell viability). Cytotoxicity of EDTA was dependent on its concentration and exposure time. Only after 60 min, considerable toxicity by EDTA was seen at concentrations higher than used in eye drops. PVA and methylparaben were not cytotoxic at any tested concentrations, even after 60 min of exposure.

Gamma-CD was nontoxic in MTT test, but cytotoxicity of other CDs was dependent on their concentrations and exposure times (Fig. 2, Table I). Viabilities of HCE cells in the presence of hydrophilic β-CD derivatives, HP-β-CD and $(SBE)_{7m}$-β-CD, were 89% and 74% at concentrations of 50 mM after 5 min treatment, respectively, but the cell viabilities were reduced to 59% and 16% in the presence of 100 mM concentration and at longer exposure times of CDs. A more lipophilic β-CD derivative, DM-β-CD, was toxic at concentrations of ≥50 mM after 5 min treatment. The limited concentration of toxicity for α-CD was 50 mM at all exposure times, while toxicity of the tested β-CD solution (8 mM) was low (85–90% cell viability) after all exposure times. However, testing of β-CD was limited by its poor aqueous solubility (16 mM).

*Propidium Iodide Assay*

Dipivefrin induced time and concentration dependent membrane damage. Dipivefrin was cytotoxic at concentrations ≥7 mM (0.25%) even after 5 min exposure. Pilocarpine and timolol did not show membrane damaging effects. Dexamethasone (25 mM, 1%) affected the cell membrane integrity damaging 25% of the cells in 5 and 60 min (Fig. 3).

BAC damaged 40% of the cells at concentrations of ≥0.28 mM (0.01%) after 5 min exposure (Fig. 3), but 60 min exposure killed all the cells. Methylparaben, EDTA and PVA did not cause membrane damage.

Gamma-CD did not cause decreased cell viability. HP-β-CD and $(SBE)_{7m}$-β-CD induced minor (10–15%) membrane damage which was only slightly affected by the exposure time and the dose. Cytoxicities of DM-β-CD and α-CD were dependent on CD concentration and exposure time (Fig. 4). Toxic concentration of α-CD was 50 mM in this assay. After 5 min exposure DM-β-CD was nontoxic, but toxicity was increased with increasing exposure time at concentrations of 50 mM and above.

$IC_{50}$ values for cell viability from propidium iodide and MTT tests were collected in Table I. The MTT and propidium iodide assays gave similar results for BAC, α-CD and dipivefrin (Table I). In the propidium iodide assay their $IC_{50}$ values at 5 min were higher than those at 30 and 60 min and higher than

**Table I.** $IC_{50}$ Values (Mean ±SD, n=2) Obtained in the MTT and Propidium Iodide Cytotoxicity Assays

| | MTT $IC_{50}$ (mM) | | | Propidium iodide $IC_{50}$ (mM) | | |
|---|---|---|---|---|---|---|
| Compound | 5 min | 30 min | 60 min | 5 min | 30 min | 60 min |
| BAC | 0.17 ± 0.01 | 0.15 ± 0.01 | 0.13 ± 0.02 | 0.59 ± 0.12 | 0.19 ± 0.01 | 0.17 ± 0.003 |
| EDTA | -* | -* | -* | -* | -* | -* |
| Methylparaben | -* | -* | -* | -* | -* | -* |
| PVA | -* | -* | -* | -* | -* | -* |
| Dipivefrin | 5.1 ± 1.1 | 3.6 ± 0.2 | 2.7 ± 0.1 | 19.4 ± 5.2 | 7.7 ± 1.4 | 6.8 ± 0.5 |
| Timolol | -* | 42 ± 17 | 24 ± 4 | -* | -* | -* |
| Pilocarpine | -* | 172 ± 10 | 113 ± 3 | -* | -* | -* |
| Dexamethasone | -* | -* | -* | -* | -* | -* |
| α-CD | 36 ± 3 | 33 ± 3 | 28 ± 1 | 81 ± 5 | 36 ± 1 | 25 ± 1 |
| DM-β-CD | 41 ± 2 | 24 ± 9 | 14 ± 4 | -* | -* | -* |
| $(SBE)_{7m}$-β-CD | -* | 79 ± 2# | 77 ± 1# | -* | -* | -* |
| HP-β-CD | -* | -* | -* | -* | -* | -* |
| γ-CD | -* | -* | -* | -* | -* | -* |

*Note:* # Solution was hypertonic. * Cell viability is not reduced to 50% of control. $IC_{50}$=test agent concentration that decreases cell viability by 50%.

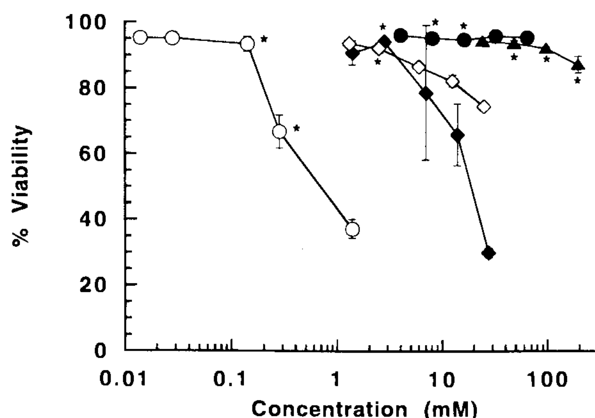


**Fig. 3.** HCE cell viability when exposed 5 min to BAC (○), dipivefrin (♦), timolol (●), pilocarpine (▲) and dexamethasone (◇) assessed by propidium iodide fluorescence (mean ±SD, n = 8). Typical concentrations of drugs in eye drop preparations have been marked with *.

$IC_{50}$ values in the MTT assay at 5 min. Membrane damage by these test substances may be seen rapidly but diffusion of propidium iodide into the nuclei at high enough concentration takes more time. Therefore, an interval of 15 min may be more valid in the propidium iodide assay. For other test substances propidium iodide assay proved to be less sensitive compared to the MTT assay.

## DISCUSSION

The toxic effect of a chemical compound *in vivo* depends on its concentration at the site of action and on the time for which the active concentration is maintained there. Residence of topical ocular drugs and excipients on the cornea is short (12). Solution drainage and systemic conjunctival absorption are the main factors decreasing the concentration of the topical ocular compounds on the corneal surface (13). In cell culture systems these factors are lacking and, therefore, the toxicity of tested compounds *in vivo* may be overestimated in cell culture. Although, *in vitro* cell culture is different from *in vivo* situation it could be useful in predicting corneal toxicity and in evaluating its mechanisms.

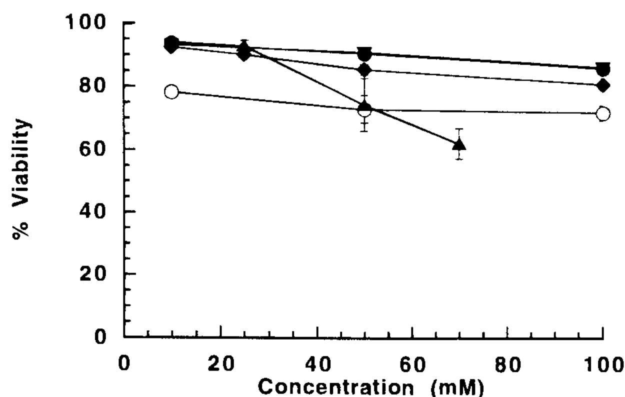


**Fig. 4.** Cytotoxicity of α-CD (▲), γ-CD (●), DM-β-CD (▼), HP-β-CD (♦) and $(SBE)_{7m}$-β-CD (○) to HCE cells in the propidium iodide assay after 5 min exposure time (mean ±SD, n = 8).

Value of the corneal epithelial cell line in toxicity testing can be estimated by comparing the results with *in vivo* situation. In commercial eye drops, the clinically used concentrations of dipivefrin, pilocarpine and timolol are generally well-tolerated. Clinically used concentrations were not toxic at short contact times in cell culture but toxic effects were elicited after 60 min.

In cell culture 2.8 mM (0.1%) dipivefrin was well tolerated, but long term use of 2.8 mM dipivefrin eye drops *in vivo* may cause abnormal proliferation of epithelial cells (14).

Pilocarpine was nontoxic in cell culture at concentrations below 48 mM (1%) after 5 min exposure, but toxicity was increased gradually with increasing exposure time and higher doses. Higher concentrations of pilocarpine (96 mM, 2%) have induced loss of microvilli and wrinkling of plasma membrane of rabbit corneas studied after 20 min of application with a scanning electron microscope (15). *In vivo* controlled release of pilocarpine (40 μg/hour) from Ocusert® is well tolerated (16). The resulting concentrations in tear fluid are, however, less than 10 mM.

Ocular irritation in patients taking commercial timolol eye drops (8 mM and 16 mM) has also been reported (17), but high concentration of BAC (0.28 mM, 0.01%) in the eye drop vehicle may in part explain the observed irritation (18). These timolol concentrations caused minor cell death *in vitro* after 5 min exposure, and after 1 h exposure the cell viability was 70–80%. In general, timolol is considered to be safe on the cornea. This is in line with the cell culture results (Fig. 1, 4).

Dexamethasone was nontoxic in the MTT test, even though it seemed to interact with the cell membrane determined by the propidium iodide assay. Low dexamethasone concentration (2.9 μg/ml, 7.4 μM) released from an intraocular device during 10 weeks showed no evidence of toxicity in rabbit eyes (19).

We observed that concentration of BAC in some commercial eye drops (0.28 mM, 0.01%) shows significant toxicity even after 5 min exposure time, while the most common concentration (0.11 mM, 0.004%) was only slightly toxic. Toxicity of BAC to many cell types is well documented (7,8,18,20). Toxic concentrations have varied from 0.003 mM to 0.28 mM depending on the experimental conditions. Although BAC did not show significant acute irritation in rabbit blinking assay (21), it is known that in clinical practice BAC may interfere with the corneal epithelium. This is an important reason for the increased use of preservative free unit dose eye droppers. Tests with the corneal epithelial cells showed the cytotoxicity of BAC at relevant concentrations.

The other pharmaceutical excipients of eye drops were safe up to 1 h of exposure at concentrations normally used in eye drops. Methylparaben (2.6 mM, 0.04%) and EDTA (2.7 mM, 0.1%) are also nonirritating *in vivo* in rabbits (21), while laser scanning confocal microscope studies with rabbit corneas revealed that EDTA (0.01-1 mM) causes membrane damage due to its effects on cell cytoskeleton (22). Impedance measurements showed that corneal resistance and capacitance were significantly decreased in the presence of 1 mM EDTA at exposure times of ≥60 min (21). In our experiments EDTA (1–5 mM) caused significant toxicity only after exposure of 60 min.

Taking into account the short contact time of topically applied drugs with ocular surface, γ-CD, HP-β-CD and $(SBE)_{7m}$-β-CD seem to be relatively safe on the corneal epithelium. High osmotic pressure of 100 mM $(SBE)_{7m}$-β-CD solution may partly contribute to its ocular toxicity after 1 h exposure.

Its toxicity was greater than that of hypertonic mannitol solution. Control solution was, however, chemically different since mannitol is neutral and $(SBE)_{7m}$-β-CD is negatively charged. In terms of the corneal toxicity HP-β-CD and $(SBE)_{7m}$-β-CD seem to be approximately equal. Toxic effects of α-CD and DM-β-CD exhibited very rapidly. Harmful membrane interactions of DM-β-CD have been generally known (23,24), but surprisingly high toxicity was obtained with α-CD. This might be due to its high potency to extract phospholipids from membranes (25). Recently, its penetration enhancer properties were studied with excized porcine and bovine corneas. The corneal permeability of pilocarpine increased significantly in the presence of 82 mM (8.54%) α-CD solution, which raised a question of its possible damaging effects on the cornea (26). Cytotoxicity of CDs may be due to the release of membrane components such as proteins, cholesterol, and phospholipids via complexation with CDs.

The mitochondrion-based MTT assay was a more sensitive indicator of toxicity in corneal epithelial cells than the plasma membrane-based propidium iodide assay. This is based on the fact that the MTT test clearly showed toxicity at higher concentrations than those in clinical use. The MTT test is an indirect method to measure cell viability, which measures the impairment of mitochondrial energy metabolism. The propidium iodide test monitors directly the collapse of plasma membrane permeability barrier, which is essential to cell function and viability. At lower concentrations, a chemical can pass through the plasma membrane without affecting the plasma membrane integrity. Mitochondria may be more sensitive to the membrane interactions of chemicals than the plasma membrane. Mitochondria contains two highly specialized membranes, an outer membrane and an inner membrane. Due to the channel-forming protein, porin, locating in the outer membrane, it is permeable to all molecules of 5000 daltons or less. Chemicals can therefore enter the intermembrane space freely and disturb the function of respiratory chain enzymes embedded in the inner membrane (27). Packing of the lipid bilayer of mitochondrial membranes may be different than that of plasma membrane because of abundance of non-bilayer forming lipids, such like cardiolipin and phosphatidylethanol amine, and because of low cholesterol content (28).

Comparison of the results with HCE cells and other cells is difficult due to the different experimental conditions (e.g. cell densities, exposure times, *in vitro* tests, different test substances). It is, however, possible to compare the results of HCE cells in MTT assay with those of primary rabbit corneal epithelial cells (7). Immortalized HCE cells seemed to be more resistant to toxic effects of BAC than the primary cells. In contrast, the cells of the three-dimensional human based tissue culture model ($SKIN^2ZK1200$) for assessment of eye irritation *in vitro* were less sensitive to the toxicity of BAC (29). Based on our findings HCE cells approximately predict the concentrations that are tolerated *in vivo*. Sensitivity of HCE cells is intermediate between primary corneal epithelium cells and $SKIN^2ZK1200$.

In conclusion, the immortalized corneal epithelial cell line is suitable for rapid toxicity screening of ocular drugs and pharmaceutical excipients. Most reliable results are obtained when assays with different endpoints are used as a battery because each test measures a specific endpoint. This may reduce the number of false positives or negatives, and makes it possible to get information of the mechanisms by which the drugs exert their toxicity.

## ACKNOWLEDGMENTS

CyDex, Inc. is gratefully acknowledged for supplying the $(SBE)_{7m}$-β-CD. Authors are grateful to Mrs. Elisa Toropainen, Ms. Marika Ruponen, Mr. Petri Nykvist, and Mr. Jonne Naarala for expert help during this study. This work was supported by grants from the Finnish Cultural Foundation, University Pharmacy, and the Julia von Wendt Foundation (P S-S); and from the Academy of Finland and the Technology Development Centre (Finland).

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