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Acute cytotoxic effects of marketed ophthalmic formulations on human corneal epithelial cells

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ABSTRACT

The purpose of the study was to devise a fast, reliable and sensitive cell viability assay for assessment of acute cytotoxicity on human corneal epithelial cells by using a clinically relevant exposure time. Acute cytotoxic effects of the pharmaceutical excipients benzalkonium chloride (BAC), macrogolglycerol hydroxystearate (MGHS40), polysorbate 80 (PS80) and marketed ophthalmic formulations (Lumigan®, Monoprost®, Taflotan®, Travatan®, Xalatan®) containing these excipients were tested. Human corneal epithelial cell (HCE-T) viability was assessed by measuring the reduction of resazurin to highly fluorescent resorufin. Expression of the tight junction proteins in HCE-T cells were characterized by immunofluorescence staining. Presence of tight junction proteins in HCE-T cells was demonstrated. BAC preserved ophthalmic formulations showed concentration-dependent and time-dependent cytotoxicity to human corneal epithelium. In contrast, no acute cytotoxicity of non-ionic stabilizing/solubilizing excipients (MGSH40 and PS80) or ophthalmic formulation containing these excipients was observed. Marketed ophthalmic formulations used for glaucoma medication show differential toxicity on human corneal epithelial cells. The present study revealed that BAC-preserved ophthalmic formulations were able to induce acute cytotoxic effects even during a clinically relevant exposure time, which was not observed with MGSH40 and PS80 excipients or ophthalmic formulations containing these excipients.

1. Introduction

Preservatives and their allergic and toxic effects in ophthalmic formulations have been studied for the last decade. The most common preservative used in ocular products is benzalkonium chloride (BAC), which is still used in almost 70% of multi-dose ophthalmic drops (Freeman and Kahook, 2009). However, there is strong evidence from both preclinical *in vivo* experiments (Ichijima et al., 1992; Noecker et al., 2004; Uematsu et al., 2015) and patients (Hätinen et al., 1985; Uusitalo et al., 2010), suggesting BAC-induced corneal epithelial cell damage. Therefore, the pharmaceutical industry is gradually moving away from traditional preservatives, either to newer preservatives or preservative-free unit-doses (Freeman and Kahook, 2009). Consequently, effects of pharmaceutical excipients (such as preservatives, stabilizers and solubilizing agents) in ophthalmic drops on corneal epithelial cells has recently attracted renewed attention (Pauly et al., 2009; Kahook and Ammar, 2010; Ammar et al., 2011; Paimela et al., 2012; Xu et al., 2013; Smedowski et al., 2014; Shen et al., 2015). However, it remains controversial whether newer preservatives, stabilizers and solubilizing agents, such as polyquaternium-1, macrogolglycerol hydroxystearate (MGHS40) and polysorbate 80 (PS80) cause corneal cytotoxicity (Paimela et al., 2012; Smedowski et al., 2014; Shen et al., 2015) or not (Ammar et al., 2011; Xu et al., 2013; Uematsu et al., 2011; Onizuka et al., 2014).

Abbreviations: BAC, benzalkonium chloride; MGHS40, macrogolglycerol hydroxystearate; PS80, polysorbate 80; HCE-T, human corneal epithelial cell line; HBSS, Hank's balanced salt solution; DPBS, Dulbecco's phosphate buffered saline; DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride; RFU, relative fluorescence units; IC_{50} value, concentration of compound giving 50% cell viability.

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One challenge faced in drug discovery is the selection of physiologically and clinically relevant end points. Notably, exposure times employed in several preclinical *in vitro* studies greatly exceeded the actual duration of topical ophthalmic drop application in patients (Tressler et al., 2011). Yet, the concentration of the ophthalmic drug decreases rapidly after topical application due to the dilution by tear fluid and drainage mechanisms (Middleton et al., 1990). Therefore, the translational value of these findings from preclinical studies with extended exposure times for clinical practice has been questioned (Tressler et al., 2011).

In the preclinical stage, it is of critical importance to evaluate the acute toxic effects of new chemical entities as well as pharmaceutical excipients on corneal epithelial cells by using clinically relevant exposure times. The aim of this study was to devise a fast, reliable and sensitive cell viability assay for assessment of acute cytotoxicity on human corneal epithelial cells by using clinically relevant exposure time. In addition, acute cytotoxic effects of different marketed ophthalmic formulations, used for glaucoma medication, on human corneal epithelial cells were compared.

2. Materials and methods

2.1. Ophthalmic formulations and pharmaceutical excipients

Tested concentrations of active pharmaceutical ingredients and pharmaceutical excipients of the commercial products are listed in Table 1. BAC was used as a positive reference control. The non-ionic stabilizing/solubilizing excipients MGHS40 and PS80 were selected, as these are the main excipients in the ophthalmic formulations studied. In order to assess the dilution effect by tear fluid and drainage *in vitro*, the ophthalmic formulations and tested pharmaceutical excipients were diluted in Hank's balanced salt solution (HBSS, Gibco, Life Technologies) as defined in Table 1.

2.2. Cell culture

Human corneal epithelial cell line (HCE-T)(Araki-Sasaki et al., 1995) was obtained from RIKEN cell bank (Tsukuba, Japan). HCE-T cells were grown in DMEM/F12 (1:1), 5% fetal bovine serum, 100 U/ml penicillin–100 μg/ml streptomycin, 5 μg/ml insulin, 10 ng/ml human recombinant epidermal growth factor and 0.5% dimethyl sulfoxide all from Life Technologies (Carlsbad, CA, USA) in 5% CO_2 at 37 °C. Cells were seeded at 50,000 cells/cm^2 in 96-well plates (Greiner Bio-One, Kremsmünster, Austria) and on chamber slides (Nunc™, Thermo Fisher Scientific, Waltham, MA, USA). Cells were grown for three days.

2.3. Immunofluorescent staining of tight junctions

Cells were gently washed twice with Dulbecco's phosphate buffered saline (DPBS, Gibco, Life Technologies), fixed with methanol at −20 °C for 10 min, blocked and permeabilized with 5% normal goat serum (NGS, Biowest, Nuaillé, France), 0.1% Triton X-100 (Sigma-Aldrich) in DPBS for 20 min. Cells were incubated with rabbit anti-ZO-1 (1:50) or rabbit anti-occludin (1:50) (Zymed Laboratories, San Francisco, CA, USA) for 1 h 30 min. After washing with 0.5% NGS in DPBS, cells were incubated with Alexa Fluor® 488 goat anti-rabbit (1:500) (Thermo Fisher Scientific) for 1 h. Nuclei were stained with 0.1 μg/ml 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) for 5 min. The fluorescent images were obtained with fluorescence microscope (Axio Imager with ApoTome.2, Carl Zeiss, Oberkochen, Germany) and analyzed with ZEN imaging software (Carl Zeiss).

2.4. Cell viability assay

Cells were gently washed twice (100 μl/well) with DPBS to remove traces of culture medium. Then cells were exposed (50 μl/well) to ophthalmic formulations, pharmaceutical excipients and dilutions thereof (described in Table 1) at 37 °C for 10 min and 30 min. Solutions were aspirated and cells were gently washed twice (100 μl/well) with DPBS. Resazurin in HBSS (0.01 mg/ml) (Perrot et al., 2003) was added (100 μl/well). Cells were incubated with resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) at 37 °C for 2 h. Fluorescent metabolite resorufin (7-Hydroxy-3H-phenoxazin-3-one) was measured at 570 nm (ex), 590 nm (em)(Perrot et al., 2003) by using Cytation 3 multi-mode reader (BioTek Instruments, Winooski, VT, USA). Assays were conducted in quadruplicate and three to seven independent experiments were performed. The relative fluorescence units (RFU) of background was measured and deducted from all treated and control wells. Cell viability (% of control) was calculated $(RFU_{treated}/RFU_{control}) \times 100$.

Table 1
Tested concentrations of active pharmaceutical ingredients and pharmaceutical excipients.

Commercial product	Supplier	Active pharmaceutical ingredient	Pharmaceutical excipient(s)	Concentrations tested
BAC	Sigma-Aldrich (St. Louis, MO, USA)	none	BAC	0.02, 0.01, 0.005, 0.003[a], 0.002 and 0.001% (w/v)
Kolliphor® RH 40	Sigma-Aldrich	none	MGHS40	10, 3, 1, 0.3, 0.1, 0.03% (v/v)
Tween® 80	Sigma-Aldrich	none	PS80	10, 3, 1, 0.3, 0.1, 0.03% (v/v)
Lumigan®	Allergan (Irvine, CA, USA)	bimatoprost (0.3 mg/ml)	BAC (0.05 mg/ml)	Commercial product as supplied (100%). 50, 30, 10, 3, 1 and 0.3% (v/v) dilutions of the original product.
Xalatan®	Pfizer (New York, NY, USA)	latanoprost (50 μm/ml)	BAC (0.2 mg/ml)	Commercial product as supplied (100%). 50, 30, 10, 3, 1 and 0.3% (v/v) dilutions of the original product.
Monoprost®	Laboratoires Théa (Clermont-Ferrand, France)	latanoprost (50 μm/ml)	MGHS40 (50 mg/ml)	Commercial product as supplied (100%). 50, 30, 10, 3, 1 and 0.3% (v/v) dilutions of the original product.
Travatan®	Alcon Laboratories (Hünenberg, Switzerland)	travoprost (40 μm/ml)	MGHS40 (2 mg/ml), polyquaternium-1 (10 μg/ml)	Commercial product as supplied (100%). 50, 30, 10, 3, 1 and 0.3% (v/v) dilutions of the original product.
Taflotan®	Santen (Osaka, Japan)	tafluprost (15 μm/ml)	PS80 (concentration not available on the summary of product characteristics)[b]	Commercial product as supplied (100%). 50, 30, 10, 3, 1 and 0.3% (v/v) dilutions of the original product.

[a] Tested 10 min only.
[b] Highest concentration present in U.S. Food and Drug Administration (FDA) approved ophthalmic solution is 1% (U.S. Food and Drug Administration, 2016). BAC, benzalkonium chloride; MGHS40, macrogolglycerol hydroxystearate; PS80, polysorbate 80.