Note

Preserved and Unpreserved 12 Anti-allergic Ophthalmic Solutions and Ocular Surface Toxicity: In Vitro Assessment in Four Cultured Corneal and Conjunctival Epithelial Cell Lines

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In the present study, we evaluated the cytotoxicity of anti-allergic ophthalmic solutions in cultured corneal and conjunctival cells, namely SIRC (rabbit corneal epithelium), BCE C/D-1b (bovine corneal epithelial cells), RC-1 (rabbit corneal epithelium), and Chang (human conjunctival cells). The viability of cell cultures was determined following the exposure of cells to 12 commercially available anti-allergic ophthalmic solutions for varying exposure times and at various dilutions using the MTT and neutral red assays. The cell viability score (CVS) was used to compare the toxicity of different drugs. Based on CVS data, the order of cell viability after exposure to the drugs was Zepelin ≥ Tramelas PF ≥ Cumarol PF ≥ Ketotifen PF ≥ Eyevinal = Fumarton ≥ Cumarol > Intal ≥ Rizaben ≥ Tramelas ≥ Patanol Livostin. In conclusion, cell viability was mostly affected by the concentration of benzalkonium chloride rather than the active component and/or the anti-allergic action of the drug. The CVS was useful in comparing the toxicity of different drugs.

Key words: Benzalkonium chloride/Toxicity/Preservative/Eye drop/Cell viability score.

Acute or chronic allergic conjunctivitis, including vernal and atopic keratoconjunctivitis, is a common eye disease with uncomfortable manifestations that afflicts many people. Topical medications are a major form of treatment that can be continued for several months or even a year. Therefore, the long-term adverse effects of ophthalmic solutions need to be evaluated. Significant numbers of patients with allergies have ocular surface disorders that may exacerbate ocular irritation and cellular damage when anti-allergic ophthalmic solutions are used. Preservatives are known to contribute to topical drug toxicity (Pfister and Burstine, 1976; Burstine and Klyce, 1977; Baudouin et al., 2010). In addition, anti-allergic components may be toxic to ocular surface cells because some of these compounds have an impact on cell activity (e.g. by inhibiting the proliferation, migration, and other bioactivity of inflammatory and other cells; Oshima et al., 2000). In the present study, we evaluated the cytotoxicity of anti-allergic ophthalmic solutions in vitro using corneal and conjunctival cells.

The commercially available cell lines used in the present study were RC-1 (rabbit corneal epithelium; JCRB-0246; Health Science Research Resource Bank, Osaka, Japan), SIRC (rabbit corneal epithelium; CCL-60; American Type Culture Collection (ATCC), Manassas, VA, USA), BCE C/D-1b (bovine corneal epithelial cells) (JCRB-9129; Health Science Research Resource Bank), and Chang conjunctiva (human conjunctival cells; CCL-20.2; ATCC). All cell lines were cultured according to standard protocols...
provided by the manufacturers.

A 100-μL aliquot of cell medium containing approximately 2 × 10⁴ cells was harvested from a 96 well microplate (NUNC™ 167008, Thermo Fisher Scientific, Denmark) and cells were incubated for 2 days. The various ophthalmic solutions to be tested were diluted with culture media and added to the cells in culture at final concentrations equivalent to 1-, 2-, and 10-fold dilutions. Cell viability was determined after 10, 30, and 60 min exposure of cells to the test drugs using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, Tokyo, Japan) assay and with the neutral red assay after the culture medium had been replaced with fresh medium and cells had been incubated for a further 48 h. At the conclusion of the assays, absorbance was read on a spectrophotometer (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA) and the cell viability in test solutions was calculated as a percentage of the control cell viability (in medium only). Experiments were repeated eight times and the results are presented as the mean ± SD.

Table 1 and Figure 1 list the drugs evaluated in the present study, as well as their anti-allergic actions. Four of the drugs evaluated (i.e. Zepelin, Cumolol PF, Ketotifen PF, and Tramelas PF) were benzalkonium chloride (BAK) free.

The viability of all four cell lines in the different test solutions is shown in Figures 2-5. The cell viability remarkably increased in the solutions with a 10-fold dilution. Eye drops had substantial toxicity even after 10 minutes exposure when undiluted and 2-fold dilution preparations were used. The general trend that the cell viability depended on the concentration of BAK contained in the solution was noted. The viability of SiRC cells as determined by the MTT assay after 60 min exposure to a 2-fold dilution of drugs that inhibited the release of histamine from mast cells was, for example, 21.3% for Rizaben, 6.0% for Tramelas, 49.1% for Tramelas PF, 56.7% for Intl, 70.6% for Cumorol, 103.3% for Cumorol PF, 63.0% for

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Active component</th>
<th>Preservative</th>
<th>Action</th>
<th>CVS50* (CVS50&lt;sub&gt;inh&lt;/sub&gt;+CVS50&lt;sub&gt;mtt&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zepelin (Wakomo, Tokyo, Japan)</td>
<td>Acitazanolast (0.1%)</td>
<td>CB 0.25%, PM 0.026%, PP 0.014%</td>
<td>IR</td>
<td>24 (12+12)</td>
</tr>
<tr>
<td>Ketotifen PF (Nitten, Nagoya, Japan)</td>
<td>Preservative-free ketotifen fumarate (0.05%)</td>
<td>No</td>
<td>IR and HRB</td>
<td>19 (12+8)</td>
</tr>
<tr>
<td>Cumorol PF (Nitten)</td>
<td>Preservative-free cromoglicate (2%)</td>
<td>No</td>
<td>IR</td>
<td>21 (12+9)</td>
</tr>
<tr>
<td>Tramelas PF (Nitten)</td>
<td>Preservative-free tranilast (0.1%)</td>
<td>No</td>
<td>IR</td>
<td>22 (12+10)</td>
</tr>
<tr>
<td>Eyevinal (Banyu, Tokyo, Japan)</td>
<td>Ibudilaist (0.01%)</td>
<td>BAK 0.005%</td>
<td>IR</td>
<td>17 (9+8)</td>
</tr>
<tr>
<td>Fumarton (Nitten)</td>
<td>Ketotifen fumarate (0.05%)</td>
<td>BAK 0.005%</td>
<td>IR and HRB</td>
<td>17 (11+6)</td>
</tr>
<tr>
<td>Cumorol PF (Nitten)</td>
<td>Cromoglicate (2%)</td>
<td>BAK 0.005%</td>
<td>IR</td>
<td>16 (7+9)</td>
</tr>
<tr>
<td>Rizaben (Kissei, Matsumoto, Japan)</td>
<td>Tranilast (0.1%)</td>
<td>BAK 0.005%</td>
<td>IR</td>
<td>9 (5+4)</td>
</tr>
<tr>
<td>Tramelas (Nitten)</td>
<td>Tranilast (0.1%)</td>
<td>BAK 0.005%</td>
<td>IR</td>
<td>8 (4+4)</td>
</tr>
<tr>
<td>Intl (Astellas, Tokyo, Japan)</td>
<td>Cromoglicate (2%)</td>
<td>BAK 0.01%</td>
<td>IR</td>
<td>10 (4+6)</td>
</tr>
<tr>
<td>Patanol (Alcon, Fort Worth, TX, USA)</td>
<td>Olopatadine (0.1%)</td>
<td>BAK 0.01%</td>
<td>IR and HRB</td>
<td>7 (4+3)</td>
</tr>
<tr>
<td>Livostin (Santen, Osaka, Japan)</td>
<td>Levocabastine (0.025%)</td>
<td>BAK 0.015%</td>
<td>HRB</td>
<td>0 (0+0)</td>
</tr>
</tbody>
</table>

The cell viability score 50 (CVS50) was calculated as the number of cell lines with viability ≥ 50% in the presence of a 2-fold dilution of the drug.

*Data show a combined CVS50, calculated as the sum of individual CVS50 values determined using the neutral red (CVS50<sub>inh</sub>) and MTT (CVS50<sub>mtt</sub>) assays.

BAK, benzalkonium chloride; CB, chlorobutanol; PP, propylparahydroxybenzoate; PM, methylparahydroxybenzoate; IR, inhibits histamine release from mast cells; HRB, histamine H<sub>1</sub> receptor blocker.
Eyevinal, and 82.5% for Zepelin. In the presence of drugs that block histamine H₁ receptors as well as histamine release from mast cells, the viability of SIRC cells was 41.6% for Fumarton, 71.4% for Ketotifen PF, 2.1% for Livostin, and 19.4% for Patanol. These results clearly indicated that the cell viability was largely affected by the presence of BAK.

Because graphic representations of cell viability indicated that differences were most evident for solutions used at a 2-fold dilution, we expressed results as a cell viability score (CVS) to enable easy comparison of the effects of different drugs at a 2-fold dilution. Specifically, the CVS<sub>50</sub> was determined as the number of cell lines with a viability ≥ 50% after exposure to a 2-fold dilution of the drug. This concept is similar to the MIC<sub>50</sub> (i.e. the minimum inhibitory concentration of a drug required to inhibit the growth of 50% of organisms). Using the CVS<sub>50</sub> as an indicator of cytotoxicity, the order of cell viability after exposure to the different solutions was Zepelin ≥ Tramelas PF ≥ Cumorol PF ≥ Ketotifen PF ≥ Eyevinal = Fumarton ≥ Cumorol > Intal ≥ Rizaben ≥ Tramelas ≥ Patanol > Livostin (Table 1).

Examining cell viability as a function of the concentration of BAK in each of the test solutions, cell viability was found to depend mostly on the concentration of BAK, the drug concentration, and exposure time rather than on the active components in the solution and/or the anti-allergic actions of the drugs. When the CVS<sub>50</sub> values were compared, eye drops containing 0.005% BAK as the preservative were shown to be less toxic than tranilast-containing eye drops (i.e. Rizaben and Tramelas).

In the present study, we evaluated the cytotoxicity of anti-allergic eye drops by examining cell viability in multiple cell lines using different drug concentrations and exposure times. The CVS was demonstrated to be useful for the comparison of the cytotoxicity of different ophthalmic solutions, as evidenced by comparable results obtained with the CVS<sub>50</sub> and the two bioassays (MTT and neutral red). Nakashima et al. (2008) examined drug toxicity using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay in rabbit corneal
FIG. 2. Effects of undiluted and 2- and 10-fold dilutions of anti-allergic ophthalmic solutions on the viability of cultured rabbit corneal epithelial cells (RC-1) after 10, 30, and 60 min exposure, as determined using the 3-(4,5-dimethyl-2 thiazoyl) -2,5-diphenyl-2H-tetrazo-lium bromide (MTT) and neutral red (NR) assays. The cell viability was noted to be highest in the presence of Zepelin. The cell viability generally depended on the concentration of benzalkonium chloride (BAK) contained in the solutions. Similar results were obtained for the other cell lines. Data are the mean ± SD. Lines are classified according to the concentration of BAK contained in solution; solid line = BAK free, bold line = 0.005% BAK, dotted line = 0.01% or more BAK.

FIG. 3. Effects of undiluted and 2- and 10-fold dilutions of anti-allergic ophthalmic solutions on the viability of rabbit corneal epithelial cells (SIRC) after 10, 30, and 60 min exposure, as determined using the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl -2H-tetrazolium b-romide (MTT) and neutral red (NR) assays. Data are the mean ± SD.

epithelium following 24 h exposure to undiluted drugs and reported the same order of toxicity as determined in the present study using the CVS (i.e. Zepelin > Eyevinal > Zaditen (same as Fumorton) > Intal > Rizaben > Livostin). The order of toxicity was inversely correlated with the BAK concentration in the solutions, except for tranilast-containing solutions. They were more toxic than other eye drops containing 0.005% BAK as the preservative and this may be because tranilast has unique actions, including an antiproliferative effect (Oshima et al., 2000) and an anti-transforming growth factor-β effect (Yasukawa...
FIG. 4. Effects of undiluted and 2- and 10-fold dilutions of anti-allergic ophthalmic solutions on the viability of BCE C/D-1b (bovine corneal epithelial cells) after 10, 30, and 60 min exposure, as determined using the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and neutral red (NR) assays. Data are the mean ± SD.

FIG. 5. Effects of undiluted and 2- and 10-fold dilutions of anti-allergic ophthalmic solutions on the viability of cultured human conjunctival cells (Chang conjunctiva) after 10, 30, and 60 min exposure, as determined using the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and neutral red (NR) assays. Data are the mean ± SD.

et al., 2002; Song et al., 2005). On the contrary, cells in Eyevinal containing ibudilast exhibited the highest viability of the eye drops with 0.005% BAK and this may be because ibudilast has cytoprotective effects (Tominaga et al., 1996; Barkhof et al., 2010). There are two reports (Lee et al., 2008; Li et al., 2010) that olopatadine had lower toxicity than ketotifen; however, their protocol was different from the present experiment in concentration or preservatives.

Anti-allergic medications have specific actions on cells; specifically, they inhibit histamine release from mast cells, leukotriene release from lymphocytes, and the chemotaxis of inflammatory cells. In addition, they can inhibit the actions of histamine, leukotriene, and the platelet-activating factor. However, the present study does not provide any evidence of a correlation between cytotoxicity and drug action. For example, drugs containing the H₁ receptor antagonists
ketotifen, olopatadine, or livocabastine did not have distinct effects compared with solutions containing drugs that did not contain H<sub>1</sub> receptor antagonists. Pauly et al. (2007) reported that the preservatives used in anti-allergic ophthalmic solutions may interfere with the anti-inflammatory effects of the active component in conjunctival epithelial cells. Therefore, interactions between the active components and preservatives may affect the pharmaceutical actions, as well as the cytotoxicity, of the ophthalmic solutions.

Some patients with allergies have dry eye, with decreased tear drainage. In these patients, instillation of an ophthalmic solution may result in prolonged exposure of the ocular surface to the drugs, possibly leading to epithelial cell damage (Burstein et al. 1985). When considering the long-term use of ophthalmic solutions of anti-allergic drugs, clinicians should keep in mind the possible adverse effects of the preservatives in these solutions on the ocular surface.

**REFERENCES**


