Cell Viability Score as an Integrated Indicator for Cytotoxicity of Benzalkonium Chloride-Containing Antiglaucoma Eyedrops

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We evaluated the in vitro cytotoxicity of benzalkonium chloride (BAK)-containing antiglaucoma eyedrops. We prepared cell cultures of SIRC, BCE C/D-1b, RC-1, and Chang conjunctiva. The viability of cell cultures was determined using the MTT and neutral red assays. The cell viability score (CVS) was used to compare the toxicity of test solutions. %CVS50 and %CVS40/80 of each eyedrop solution were 71 and 26 for Lumigan® (0.002% bimatoprost with 0.005% BAK), 100 and 99 for Tapers® (0.0015% tafluprost, a new formula from 2010 with 0.001% BAK), 39 and -29 for 2% Trusopt® (2% dorzolamide with 0.0075% BAK), 28 and -43 for Xalacom® (latanoprost/0.5% timolol with 0.02% BAK), 88 and 66 for DuoTrav® (travoprost/0.5% timolol with no BAK), 36 and -35 for Cosopt® (2% dorzolamide/0.5% timolol with 0.0075% BAK) and 53 and -1 for Combigan® (0.15% brimonidin/0.5% timolol with 0.005% BAK). Only Xalacom® and Tapers® did not show an apparent decrease in %CVS as compared to the corresponding concentration of BAK. In conclusion, the cytotoxicity of tested eyedrops was dependent on BAK. Only the eyedrops containing latanoprost or tafaluprost showed a reduction in the cytotoxicity of BAK.

Key words: Benzalkonium chloride/Toxicity/Eye drops/Cell viability score/Glaucoma.

Recently, clinical ophthalmology in glaucoma medical therapy has two trends. One is to reduce the toxic side effects of benzalkonium chloride (BAK) and the other is to consider a fixed combination of drugs as an important therapeutic option such as Cosopt® (Strohmaier et al., 1998; Boyle et al., 1998), Xalacom® (Miglior et al., 2010), DuoTrav® (Liang et al., 2011; Ammar et al., 2011; Kitazawa et al., 2011), and Combigan® (Fudemberg et al., 2008; Motolko, 2008; Craven et al., 2005). They have been introduced for better compliance of patients and greater pressure reduction in a single dose.

The ocular surface cytotoxicity of eyedrops depends on the BAK contained as a preservative in experimental settings (Furrer et al., 2002; Boudoun et al., 2010). It is a dose dependent phenomenon and, consequently, clinicians and pharmaceutical companies recently prefer eyedrops with less or no BAK. BAK concentration has been reduced in Travatan® (from 0.0075% to zero in Travatan®Z, Alcon Japan, Tokyo, Japan) (Uematsu et al., 2011; Ryan et al., 2011), Tapros® (from 0.01% to 0.001%, Santen, Osaka, Japan) (Asada et al., 2010), and Hypadil® (from 0.05% to 0.002%, Kowa Soyaku, Tokyo, Japan, containing 0.25% nifedipilol) (Yamaguchi et al., 2010). As single dose formulation is available for tafaluprost and its safety has been indicated (Usitalo et al., 2008; Liang et al., 2008; Liang et al., 2011b; Pellinen et al., 2009; Brasnu et al., 2008). Since
eyedrops contained in a multi-dose bottle may become contaminated during the treatment period due to improper use by patients (Kim et al., 2008; Feghhi et al., 2008; Rahman et al., 2006; Taşlı et al., 2001), a preservative system is essential for patients' safety.

We have developed an integrated indicator of cytotoxicity obtained from a series of monolayer cell culture bioassays, the CVS (cell viability score) system. It expresses the cytotoxicity of a test solution quantitatively (Ayaki et al., 2011a). Previously we carried out a cytotoxicity assay of BAK in 11 concentrations (0.002 to 0.003%) and made a standard curve for cytotoxicity (Ayaki and Iwasawa 2011b). This quantitative method enables us to compare the cytotoxicity of each eyelid formulation and evaluate the interactive effects of the cytotoxicity of active ingredients and preservatives including BAK. In this study, we examined the cytotoxicity of seven antiglaucoma eyedrops and compared the values with that of corresponding concentrations of BAK in eyelid products we have previously examined.

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

A 100-μL aliquot of culture medium containing approximately 2 x 10^5 cells was harvested from each culture well (Falcon Multiwell, 96 wells) and incubated for two days. The culture medium was then replaced with 100 μL of undiluted, 2-fold, and 10-fold diluted doses of the test ophthalmic solutions, and the cells were incubated for 10, 30, or 60 minutes. Fresh culture medium was then substituted and the cells were further incubated for 48 h. Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich, St. Louis, MO, USA) assay and the neutral red (NR) (Wako Pure Chemical Industries, Osaka, Japan) assay. The MTT assay is a quantitative colorimetric assay to quantify mitochondrial activity as a measure of cell viability and proliferation. It detects only living cells and the signal generated is directly proportional to the number of viable cells. NR is uptaken by living cells. The assay results were measured spectrophotometrically (Benchmark microplate reader, BIO-RAD, Hercules, CA). Cell viability in test solutions was calculated as a percentage of control cell viability in medium only. The experiments were repeated 8-16 times and results are presented as the average +/- standard deviation.

Tested eyedrops were Lumigan® (0.002% bimatoprost, Senju Pharmaceutical Co., Ltd., Osaka, Japan, containing 0.005% BAK), Tapro® (0.0015 %tafluprost, a new formula from 2010, Santen Pharmaceutical Co., Ltd., Osaka, Japan, containing 0.001% BAK), Xalacom® (Pfizer Inc., Tokyo, Japan, containing 0.02% BAK), DuoTrav® (Alcon Japan Co., Ltd., Tokyo, Japan, containing no BAK), Cosopt® (Merck and Co., Whitehouse Station, NJ, USA, containing 0.0075% BAK), Combigan® (Allergan Inc., Irvine, CA, USA, containing 0.005% BAK), and 2% Trusopt® (Merck and Co., containing 2% dorzolamide and 0.0075% BAK). Four of them are fixed combinations of 0.005% latanoprost/0.5% timolol for Xalacom®, 0.004% travoprost/0.5% timolol for DuoTrav®, 2% dorzolamide/0.5% timolol for Cosopt®, and 0.2% brimonidine/0.5% timolol for Combigan®. The chemical structures of tested eyedrops are shown in Fig.1. Cosopt® has another formulation only for Japan containing 1% dorzolamide, 0.5% timolol, and 0.005% BAK.

The cell viability score (CVS) was used to compare the toxicity of the test solutions (Ayaki et al., 2011a). We set the reference point of cell viability at 50% for CVS50 and 40% and 80% for CVS40/80. The 80% viability can be recognized as the "nontoxic level" and the 40% viability as the "significantly toxic level" according to our previous experiments covering thousands of assays. The CVS50 was determined by the number of measurements for viability ≥ 50% of the control. The CVS40/80 was calculated as follows: CVS40/80 = (the number of measurements for viability value > 80%) - (the number of measurements for viability value < 40%). The total number of the measurements was 72 (3 concentrations, 3 exposure times, 4 cell lines, and 2 assays). The results were expressed as the percentages of CVS per total number of measurements (%CVS). To assess if the measured cell viability values between two drugs of interest are significantly different, scatter diagrams for cell viability of all the cell lines exposed to the drugs were illustrated, and a 2x3 chi-square test for independence was applied by counting the total number of plots showing a cell viability of 40% or less, between 40% and 80%, and 80% or more for each drug. P values of <0.05 were considered to be significant.

The cell viability after 60 min in selected eyedrop solutions, recently introduced fixed combination drugs and prostaglandin analogues, is shown in Figs.
FIG. 1. Chemical structure of eyedrops tested in the present study. Timolol maleate, latanoprost, and travoprost are components of the tested fixed-combination eyedrops.

FIG. 2. Effects of selected anti-glaucoma solutions diluted 1-, 2-, and 10-fold on the viability of cultured ocular surface cells (RC-1, SIRC, BCE C/D-1b, and Chang conjunctiva) after exposure for 60 minutes using the MTT assay. Data are means ± SD. Closed symbols indicate fixed combination eyedrops. BCE, BCE C/D-1b; Chang, Chang conjunctiva.

2 and 3. The cell viability apparently decreased with an increase in exposure time. The viability of the cell lines exposed to DuoTrav® without BAK and Tapros® with very low level (0.001%) of BAK tended to be greater than that exposed to the other drugs. The value of %CVS50 and %CVS40/80 for each eyedrop solution were 71/26 for Lumigan®, 100/99 for Tapros®, 28/-43 for Xalacom®, 88/66 for DuoTrav®, 36/-35 for Cosopt®, 53/-1 for Combigan®, and 39/-29 for 2% Trusopt®.

Figure 4 shows %CVS40/80 for different concentrations of BAK and antiglaucoma eyedrops. The %CVS40/80 values of BAK and some of antiglaucoma eyedrops are from our previous works including Xalatan®, Travatan®, Travatan® Z (Ayaki and Iwasawa, 2010), 2% Mikelan®/2% MikeranLA® (Ayaki et al., 2011c), and Hypadil® (Yamaguchi et al., 2010). When the CVS values of eyedrops are
FIG. 3. Effects of selected anti-glaucoma solutions diluted 1-, 2-, and 10-fold on the viability of cultured ocular surface cells (RC-1, SIRC, BCE C/D-1b, and Chang conjunctiva) after exposure for 60 minutes using the neutral red assay. Data are means ± SD. Closed symbols indicate fixed combination eyedrops. BCE, BCE C/D-1b; Chang, Chang conjunctiva.

FIG. 4. CVS40/80 of benzalkonium chloride (BAK) and antiglaucoma eyedrops. The values of CVS40/80 of BAK of a series of concentrations and the CVS40/80 of antiglaucoma eyedrops not tested in the present study are from our previous works (see text for references). The CVS40/80 of eyedrops is plotted against their corresponding BAK concentration. Note the relationship of the cytotoxicity of eyedrops and BAK. Most eyedrops exhibit more cytotoxicity than BAK except for Xalatan®, Xalacom®, and Tapro® (new formulation) that did not have an apparent decrease in comparison with a corresponding concentration of BAK. Square symbols = prostaglandin analogue, triangle symbols = beta blockers or carbonic anhydrase inhibitors, closed symbols = single formulation, open symbols = fixed combination.

plotted against the values of the BAK at corresponding concentrations of BAK contained in the eyedrops, a close relationship in cytotoxicity between eyedrops and the BAK is found. Table 1 also shows that %CVS40/80s of eyedrops were dependent on the concentration of BAK.

To further determine if there are any eyedrops which show a close relation with the %CVS40/80 of BAK, a 90% probability ellipse for %CVS40/80s and a series of BAK concentrations was illustrated by using JMP Pro 9 (SAS Institute, Cary, NC, USA.) (Fig. 5). Combigan®, 0.5% Timoptic®, 2% MikelanLA®, 1%
TABLE 1. %CVS40/80 for benzalkonium chloride and antiglaucoma eyedrops tested in the present and previous* studies

<table>
<thead>
<tr>
<th>Drug group</th>
<th>Name</th>
<th>Eyedrop %CVS40/80** Concentration (%)</th>
<th>Benzalkonium chlorde %CVS40/80** Concentration (%)</th>
<th>Subtraction of %CVS40/80 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandin analogue</td>
<td>Xalatan*</td>
<td>-42</td>
<td>0.02</td>
<td>-46</td>
</tr>
<tr>
<td></td>
<td>Travatan*</td>
<td>-54</td>
<td>0.015</td>
<td>-33</td>
</tr>
<tr>
<td></td>
<td>TravatanZ*</td>
<td>83</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Lumigan</td>
<td>26</td>
<td>0.005</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Tapros (-2009)*</td>
<td>-25</td>
<td>0.01</td>
<td>-15</td>
</tr>
<tr>
<td></td>
<td>Tapros (2010-)*</td>
<td>99</td>
<td>0.001</td>
<td>85</td>
</tr>
<tr>
<td>Beta blocker</td>
<td>0.5%Timoptol*</td>
<td>-13</td>
<td>0.005</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>2%Mikelan*</td>
<td>-25</td>
<td>0.005</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>2%MikelanLA*</td>
<td>-26</td>
<td>0.005</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Hypadil*</td>
<td>50</td>
<td>0.002</td>
<td>83</td>
</tr>
<tr>
<td>Carbonic anhydride inhibitor</td>
<td>1%Trusopt*</td>
<td>-10</td>
<td>0.005</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>2%Trusopt</td>
<td>-29</td>
<td>0.0075</td>
<td>-1</td>
</tr>
<tr>
<td>Fixed combination</td>
<td>Xalacom</td>
<td>-43</td>
<td>0.02</td>
<td>-46</td>
</tr>
<tr>
<td></td>
<td>DuoTrav</td>
<td>69</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Cosopt with 2%dorzolamide</td>
<td>-35</td>
<td>0.0075</td>
<td>-1</td>
</tr>
<tr>
<td></td>
<td>Cosopt with 1%dorzolamide***</td>
<td>-27</td>
<td>0.005</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Combigan</td>
<td>-1</td>
<td>0.005</td>
<td>39</td>
</tr>
</tbody>
</table>

* = references are indicated in the text. **CVS40/80 = (number of measurements for viability value >80%) – (number of measurements for viability value <40%). Total number of measurements was 72 (3 concentrations, 3 exposure times, 4 cell lines, 2 assays). Results were expressed as % of total measurements (%CVS40/80). ***Japanese formulation of Cosopt® containing 1% dorzolamide, 0.5% timolol, and 0.005% benzalkonium chloride (unpublished data).

FIG. 5. Ninety percent probability ellipse for %CVS40/80s and a series of benzalkonium chloride (BAK) concentrations. Circle, triangle, and rhombus symbols indicate BAK, eyedrops not containing and containing prostaglandin, respectively. Combigan®, 0.5% Timoptol®, 2% MikelanLA®, 2% Trusopt® and Cosopt® with 2% dorzolamide are in the lower area outside the ellipse.
Trusopt®, 2% Trusopt® and Cosopt® with 2% dorzolamide could be found on the lower area outside the ellipse. Beside the six eyedrop solutions, as shown in Fig. 4, most eyedrops except Xalatan®, Xalacom®, and Tapros® (formulation since 2010) tended to exhibit a cytotoxicity severer than BAK. The %CVS40/80s of Xalatan®, Xalacom®, and Tapros® did not show an apparent decrease as compared to those of corresponding concentrations of BAK.

Accordingly, as shown in Table 1, the differences in %CVS40/80s between eyedrops and BAK were clearly separated to two groups. The value of prostaglandin analogues ranged from +4 to -21 and those of beta blockers and carbonic anhydrase inhibitors ranged from -28 to -66. Statistical analysis by Mann-Whitney U-test confirmed that there is a significant difference ($P<0.001$) in the subtraction of %CVS40/80s between the two groups. For instance, to compare the typical eyedrops belonging to the two groups, a scatter diagram for cell viability of all the cell lines exposed to Lumigan® and 2% MikelanLA®, both of which contained 0.005% BAK, were illustrated (Fig. 6), and a 2x3 chi-square test was applied by counting the total number of plots that showed a cell viability of 40% or less, between 40% and 80%, and 80% or more for each eyedrop. The calculated $\chi^2$ for %CVS40/80 are 24.25 ($P<0.001$), indicating that the %CVS40/80 value in the Lumigan®-treated cells is significantly independent of that in the 2% MikelanLA®-treated cells.

As shown in Figs. 2 and 3, of the six eyedrops tested, Tapros® (formulation since 2010) was apparently least cytotoxic followed by DuoTrav® and Lumigan®. Since all the three eyedrops contained a prostaglandin analogue as an active ingredient, a hypothesis was raised that the cytotoxicity of prostaglandin analogue-containing eyedrops is relatively low as compared to that of non-prostaglandin eyedrops. This hypothesis led us to examine intensively the relation between active ingredients and cytotoxicity. In general, the cytotoxicity of eyedrops depends on the concentration of BAK used as a preservative. Thus, the relation was evaluated in association with corresponding concentration of BAK in eyedrops.

For this purpose, the cytotoxicity data (expressed as %CVS40/80) presented in our previous studies for Xalatan®, Travatan®, Travatan® Z (Ayaki and Iwasawa, 2010), 2% Mikelan® /2% MikelanLA® (Ayaki et al., 2011b), and Hypaflat® (Yamauchi et al., 2010) are cited in Table 1. %CVS40/80 is an index for the cytotoxicity of eyedrops reflecting the effects of exposure time, drug concentration, and differences in cell types (Ayaki et al., 2011a). As shown in Table 1 in which the differences between %CVS40/80s of eyedrops and those of corresponding concentrations of BAK were calculated, subtracted values of %CVS40/80s of prostaglandin analogue eyedrops are smaller than that those of non-prostaglandin eyedrops ($P<0.05$ assessed by the Mann Whitney U-test), suggesting that the

![P<0.001 by $\chi^2$ test](image)

**FIG. 6.** Scatter diagrams for the cell viability of all the cell lines exposed to Lumigan® and 2% MikelanLA®, both of which contained 0.005% benzalkonium chloride. Each open circle indicates a measurement of cell viability under a fixed condition including dilution (undiluted, 2-fold and 10-fold dilution) and a given exposure time. It is demonstrated that %CVS40/80 in the Lumigan®-treated cells is significantly independent of that in the 2% MikelanLA®-treated cells ($P<0.001$ by chi-square test).
cytotoxicity of prostaglandin analogue eye drops is dependent on the cytotoxicity of BAK.

Figure 4 also supports the idea that all %CVS40/80s of prostaglandin analogue eye drops are close to those of the corresponding concentrations of BAK. Values for %CVS40/80s of BAK are cited from our previous study (Ayaki and Iwasawa, 2011b). To confirm this, the 90% probability ellipse for %CVS40/80s and a series of BAK concentrations is illustrated (Fig. 5) to find eyedrops lying outside the close relation between %CVS40/80 and BAK concentrations. As a result, six products are found on the lower area outside the ellipse, indicating that these are significantly more cytotoxic than the corresponding concentrations of BAK. These are Combigan®, 0.5% Timoptol®, 2% MikelanLA®, 1% Trusopt®, 2% Trusopt® and Cosopt® with 2% dorzolamide. Since all the six eydrop products are non-prostaglandin eyedrops, it is strongly suggested that the cytotoxicity of active ingredients in these eyedrops are added to the cytotoxicity of BAK. Regarding the cytotoxicity of fixed combinations of drugs, it is complicated and the present results are not conclusive since drug interactions among active and inactive ingredients are mixed up in a single bottle although in general it seems to depend on the BAK concentration and presence of the prostaglandin analogue.

Regarding the optimal BAK concentration as a preservative in eyedrops, a preservative-effectiveness test should be conducted according to the Japanese Pharmacopoeia with various concentrations of BAK. For instance, the optimal concentration of BAK in 0.0015% tafluprost ophthalmic solution (Tapros®) was examined from the point of view of ocular safety and preservative efficacy (Asada et al., 2010). Results showed that 0.0005% to 0.003% of BAK in the tafluprost ophthalmic solution was the optimal concentration in regards to both ocular surface safety and preservative efficacy. In other words, the optimal BAK concentration should be determined for each ophthalmic solution. In some cases, BAK concentrations which cause cytotoxic effects as indicated by low CVS values are used in commercially available eyedrops such as Xalatan® and Xalacom®. Since it is empirically known that keratoconjunctival damage in clinical use is usually alleviated by washing out the eye with tears, such concentrations of BAK are sometimes used in formulations.

In conclusion, it is a remarkable finding that all %CVS40/80s of prostaglandin analogue eye drops are close to those of the corresponding concentrations of BAK. Latanoprost or tafluprost-containing eyedrops, Xalatan®, Xalacom®, and Tapros® (formulation since 2010), exhibited no differences or had greater %CVS40/80s as compared with those of corresponding concentrations of BAK. Some prostaglandin analogue eye drops have less cytotoxicity even with considerable concentrations of BAK. For example, latanoprost (in Xalatan® containing 0.02% BAK, Pfizer Inc., USA) and travoprost (Travatan® containing 0.015% BAK) have been reported to have the effect to reduce BAK toxicity (Guenoun et al., 2005). Based on the present results, it is strongly suggested that, when the concentrations of BAK in eyedrops are the same, the ocular surface cytotoxicity of eyedrops containing prostaglandin analogues does not exceed that of eyedrops containing non-prostaglandin ingredients such as beta blockers and carbonic anhydrase inhibitors. Guenoun et al. (2005) showed cytoprotective and antioxidative effects of prostaglandin analogues under such limited conditions such as 10-fold dilution and 30 min exposure. Therefore, in addition to the low cytotoxicity of prostaglandin analogues, the results obtained by our generalized protocol support the idea that this cytoprotective and antioxidative property of prostaglandin analogues could contribute to restraining the cytotoxic effect of BAK.

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