

Fluoroquinolone Eye Drop–Induced Cytotoxicity: Role of Preservative in P2X7 Cell Death Receptor Activation and Apoptosis

Mélody Dutot,¹ François Pouzaud,¹ Isabelle Larosche,¹ Françoise Brignole-Baudouin,^{1,2,3} Jean-Michel Warnet,^{1,2,3} and Patrice Rat^{1,2,3}

PURPOSE. To investigate in vitro whether eye toxicity is attributable to the preservative or the fluoroquinolone used in ophthalmic formulations.

METHODS. Corneal and conjunctival cell lines were incubated with preserved (benzalkonium chloride [BAC]) or unpreserved ofloxacin solutions for 15 minutes. Several concentrations of BAC were also tested (0.0025%–0.01%). Membrane integrity, reactive oxygen species, and superoxide anion production were assessed with the neutral red test, the 2',7'-dichlorofluorescein diacetate test, and the dihydroethidium test, respectively. P2X7 cell death receptor activation was evaluated using the YO-PRO-1 assay and apoptosis (chromatin condensation and translocation of phosphatidylserine) using the Hoechst 33342 and annexin V-FITC dyes. Tests were performed with microplate cytofluorometry, inverted fluorescence microscopy, and flow cytometry.

RESULTS. The preserved solution and all tested BAC concentrations induced a significant decrease in membrane integrity, unlike the unpreserved ofloxacin. Reactive oxygen species and superoxide anion productions observed for all solutions were significantly higher for the preserved ofloxacin and BAC solutions, which also induced apoptosis (chromatin condensation and translocation of phosphatidylserine) through P2X7 pore opening, whereas unpreserved ofloxacin did not.

CONCLUSIONS. The cytotoxicity observed with fluoroquinolone eye drops seems to be caused mainly by the preservative, which induced P2X7 cell death receptor activation associated with oxidative stress and apoptosis. Therefore, it is recommended that fluoroquinolone be used in preservative-free formulations. (*Invest Ophthalmol Vis Sci.* 2006;47:2812–2819) DOI:10.1167/iovs.06-0224

Fluoroquinolones have become important antibacterial agents in the treatment of ocular infections. Fluorinated derivatives of nalidixic acid, the first quinolone molecule (developed in 1962), showed a better antibacterial spectrum than did the quinolones. Oral fluoroquinolones were the first to be

developed in the early 1980s followed by products for systemic and topical ophthalmic use in the mid-1980s.¹ They are now widely used as antibacterial agents to treat ocular infections, with intravitreal, topical, and systemic routes of administration. Ciprofloxacin, ofloxacin, and norfloxacin have good activity against Gram-negative and -positive bacteria; thus, they are mainly used in ophthalmic antibiotic prophylaxis because of their broad-spectrum activity and good ocular diffusion. Their formulations are used to treat conjunctivitis, keratitis, and corneal ulcerations because they possess excellent in vitro effectiveness against cornea pathogens such as *Pseudomonas aeruginosa* or *viridans* group streptococci.² Fluoroquinolones are generally well tolerated, but as the treatment may be administered over a fairly long period, the risk of damaging the weakened cornea is heightened. In fact, several investigators have reported delays in the healing rate and corneal perforations after the administration of fluoroquinolone preparations. In clinical practice, data showed some corneal perforations after preserved ofloxacin treatment in eye.³ In vivo studies on rabbits showed keratocyte depletion and inflammatory infiltration on injured corneas after treatment with the commercial formulation of 0.3% ofloxacin, confirmed by a histopathological analysis (Bekoe NA, et al. *IOVS* 1999;40:ARVO Abstract S548). In vitro, delayed corneal wound healing has been observed in corneal cultures after incubation with ofloxacin at concentrations higher than 0.5 mg/mL,⁴ and cytotoxicity in corneal cells (the SIRC line) incubated with ofloxacin at concentrations higher than 1.5 mg/mL.⁵

Moreover, many eye drops contain preservatives known to cause severe side effects to the ocular surface: benzalkonium chloride (BAC) is the preservative currently used the most, and many in vivo and in vitro studies have shown its toxicity on conjunctival and corneal cells as well as its immunoallergic effects, possibly due to its slow turnover and to its high ocular retention (up to 48 hours after administration of a single drop).⁶ In clinics, BAC has been implicated in antiglaucoma drug intolerance.⁷ It has been reported that BAC induces conjunctival cell growth arrest and cellular injury at a concentration as low as 0.0001%.⁸ Morphological and physiological studies have shown differences in morphology, electrophysiology, and cornea hydration between cells treated with preserved formulations and cells treated with unpreserved formulations.⁹ Moreover, another study demonstrated contribution of quaternary ammoniums to oxidative stress and apoptosis in conjunctival cells.¹⁰

YO-PRO-1 penetration in cells is directly linked with P2X7 pore opening, which is specific to apoptosis. P2Z/P2X7 receptors are usually studied on microglial or astrocytoma cells^{11–14} and on immune system cells,^{15–19} to assess their implication in many cellular pathways, such as oxidative stress,¹⁵ MAP kinase phosphorylation,^{12,18,20} inflammation,^{17,19,21–23} and apoptosis.^{24,25} The P2X7 receptor is activated during apoptosis through extracellular adenosine triphosphate (ATP), which induces pore formation with Ca²⁺ influx.²⁶ The carboxyl-terminal domain, specific to the P2X7 receptor, is essential for the

From the ¹Laboratoire de Toxicologie, Faculté des Sciences Pharmaceutiques et Biologiques, Université René Descartes-Paris, Paris, France; ²INSERM U598, Institut Biomédical des Cordeliers, Paris, France; and ³Centre Hospitalier National d'Ophthalmologie des Quinze-Vingts, Paris, France.

Submitted for publication March 18, 2005; revised July 29 and December 27, 2005, and March 1, 2006; accepted May 2, 2006.

Disclosure: M. Dutot, None; F. Pouzaud, None; I. Larosche, None; F. Brignole-Baudouin, None; J.-M. Warnet, None; P. Rat, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Patrice Rat, Laboratoire de Toxicologie, Faculté des Sciences Pharmaceutiques et Biologiques, Université René Descartes-Paris5, 4 Av de l'Observatoire, 75270 Paris cedex 06-France; patrice.rat@univ-paris5.fr.

lytic action of ATP.²⁷ Thus, cornea perforations could be explained by the cytolytic action of BAC via P2X7 receptor activation, since Groschel-Stewart et al.²⁸ have shown P2X7 receptor localization in the corneal epithelium.

Kovoor et al.²⁹ recently observed, using confocal microscopy, that preserved fluoroquinolone solutions with BAC induced damage to corneal epithelium, whereas unpreserved solution did not, but the mechanism remained unclear. The purpose of this study was to compare and understand the toxicity mechanism of two topical preparations containing ofloxacin: one preserved with BAC and the other with no preservative, in two ocular cell lines. The role of BAC itself was also evaluated. Several cellular markers were assessed by microplate cold-light cytofluorometry, microplate cytofluorometry, inverted fluorescence microscopy, and flow cytometry. Cell viability, reactive oxygen species (ROS) overproduction, superoxide anion (O_2^-) overproduction, and apoptosis were evaluated with neutral red, 2',7'-dichlorofluorescein diacetate, dihydroethidium, YO-PRO-1, Hoechst 33342, and annexin V-FITC fluorescent probes, respectively.

MATERIALS AND METHODS

Cell Culture

A rabbit corneal cell line (SIRC, CCL 60; ATCC, Manassas, VA) and a human conjunctival cell line (Wong Kilbourne derivative of Chang conjunctiva WKD, 93120839; ECACC [European Collection of Cell Cultures], Porton Down, UK) were cultured under standard conditions (moist atmosphere of 5% CO_2 at 37°C) in Dulbecco's minimum essential medium (DMEM; Eurobio, Les Ulis, France) supplemented with 10% fetal bovine serum (Dominique Dutscher, Brumath, France), 2 mM L-glutamine (Eurobio), 50 IU/mL penicillin (Eurobio) and 50 IU/mL streptomycin (Eurobio). The SIRC cell line was validated by the European Centre for the Validation of Alternative Methods (ECVAM, Ispra, Italy; invitox protocol number 40 for ophthalmology cytotoxicity studies on corneal cells). The human conjunctival cell line was chosen to confirm P2X7 receptor activation on another ocular cell line.

Confluent cultures were removed by trypsin (Eurobio) incubation and were seeded into 96-well culture microplates (Corning, Schiphol-Rijk, The Netherlands) at a density of 60,000 (corneal cells) or 90,000 (conjunctival cells) cells/mL for analysis. Cultures were kept at 37°C for 24 hours.

Cell Incubation

When cells reached approximately 80% of confluence, the culture medium was removed and the cells were exposed to (1) two topical formulations containing 0.3% ofloxacin—that is, Oflo-BAC (Oflocet; Aventis, Romainville, France), a preservative-free solution without BAC, and Oflo+BAC (Exocine; Allergan, Irvine, CA) with BAC at a concentration of 0.0025%; (2) three different concentrations of BAC (Sigma-Aldrich, Saint Quentin Fallavier, France), dissolved in 0.9% NaCl 0.0025%, 0.005% and 0.01%; and (3) 0.9% NaCl, used as a cellular control.

Two different incubation times were tested: 15 minutes of incubation and 15 minutes of incubation followed by a 24-hour recovery period (only for the neutral red test). A recovery period served to study the eventual reversibility of the cytotoxicity.

Experimental Procedures

Experiments were conducted with microplate cold-light fluorometry, which allows fluorometric detection (280–870 nm) with high sensitivity (from picograms to femtograms per milliliter; Fluorolite 1000; Dynex, Cergy Pontoise, France)³⁰ and flow cytometry (Coulter EPICS XL-MCL; Beckman Coulter, Fullerton, CA). According to the recommendations of the ECVAM, three cellular parameters were evaluated:

cell viability, ROS production, and chromatin condensation.³¹ Another parameter was also evaluated: P2X7 cell death receptor activation.

Microplate Cold-Light Fluorometry. This technique allows the use of fluorescent probes directly on living cells and detects the fluorescent signal directly in the microplate in less than 1 minute (in a 96-well microplate).

Cell Viability and Membrane Integrity: Neutral Red Test. Membrane integrity, closely correlated with cell viability, was evaluated with neutral red (NR; Fluka, Buchs, Switzerland) using fluorometric detection (excitation, 535 nm; emission, 600 nm). Neutral red was used at a concentration of 50 μ g/mL. In accordance with the validated protocol of Borenfreund and Puerner,³² 200 μ L per well of neutral red solution was added to living cells. After a 3-hour incubation time at 37°C in moist atmosphere with 5% CO_2 , the neutral red fluorescence signal was measured.

Reactive Oxygen Species Overproduction: DCFH-DA Test. ROS were detected with the 2',7'-dichlorofluorescein diacetate probe (DCFH-DA, Invitrogen, PoortGebouw, The Netherlands), added to living cells before any drug incubation. Once inside the cell, this probe is cleaved by endogenous esterases and can no longer pass out of the cell. The de-esterified product becomes the fluorescent compound 2',7'-dichlorofluorescein after oxidation by ROS. Cells were preincubated for 20 minutes with a 20 μ M DCFH-DA solution, and after they dye was removed, the cells were exposed to the different formulations. Fluorescence detection was undertaken at 15 minutes. The fluorescent signal (excitation, 485 nm; emission, 535 nm) is proportional to ROS production.

Superoxide Anion Overproduction: Dihydroethidium Test. O_2^- was detected using dihydroethidium probe (HE; Invitrogen). It was oxidized to the fluorescent ethidium cation by O_2^- , allowing the cation to bind to nuclear DNA with an extensive fluorescence enhancement. Cells were first incubated in a dye solution at 1.58 μ g/mL for 20 minutes, as previously described for the DCFH-DA test. Fluorescence detection (485 nm; emission, 535 nm) was performed at 15 minutes.

P2X7 Cell Death Receptor Activation: YO-PRO-1 Test. YO-PRO-1, a DNA probe, is usually used to discriminate cells dying by apoptosis versus necrosis with both flow cytometry³³ and fluorescence microscopy.³⁴ This probe enters apoptotic cells only through the P2X7 receptor.

After incubation with fluoroquinolones, a 2- μ M YO-PRO-1 probe (Invitrogen) solution in phosphate-buffered saline (PBS; Dominique Dutcher) was distributed in the wells (200 μ L per well), and the microplate was placed at room temperature in the dark, for 10 minutes. The fluorescence signal was scanned using a cytofluorometer (Safire; Tecan, Lyon, France) with a small band-pass and precise wave lengths for YO-PRO-1 probe fluorescence detection (excitation, 491 nm; emission, 509 nm, band-pass, 5 nm).

The specificity of YO-PRO-1 dye toward purinergic P2X7 pore opening was confirmed with three puric bases: ATP, BzATP (2'-3'-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate) and oATP (oxidized ATP), provided from Sigma-Aldrich. The cells were incubated with oATP (50 μ M in PBS) for 2 hours at 37°C, then stimulated with 3 mM ATP (in PBS) for 15 minutes. They were also incubated with ATP (3 mM in PBS) and BzATP (50 μ M in PBS) for 15 minutes at 37°C. The YO-PRO-1 solution was then distributed in each well to allow the fluorescence signal measurements.

Apoptosis: Chromatin Condensation Assessment—Hoechst 33342 Test. Hoechst 33342 (Invitrogen) was used to evaluate chromatin condensation in cells with propidium iodide to discriminate necrotic cells. The UV fluorescent probe Hoechst 33342 (excitation, 360 nm; emission, 450 nm) enters living cells and apoptotic cells, whereas propidium iodide enters necrotic cells much faster than Hoechst 33342. The cells were exposed for 30 minutes to a Hoechst 33342 solution at a concentration of 10 μ g/mL containing 1 μ L of propidium iodide (1 mg/mL in water). They were then observed by inverted fluorescence microscopy (DMIRB; Leica, Heidelberg, Ger-

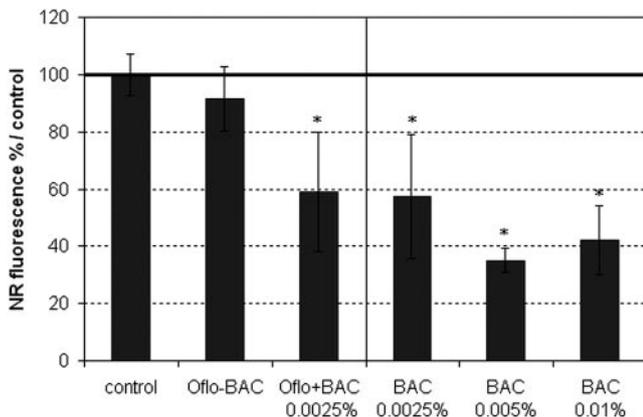


FIGURE 1. Membrane integrity evaluation by neutral red (NR) test after incubation with unpreserved or preserved ofloxacin and different concentrations of BAC. After 15 minutes of incubation, there was a significant decrease in membrane integrity with preserved ofloxacin and BAC treatments, but not with unpreserved ofloxacin. * $P < 0.001$ compared with the control.

many) and photographed (numeric coolpix 5000; Nikon, Tokyo, Japan).

Apoptosis: Translocation of Phosphatidylserine Evaluation—Annexin V Test. In apoptotic cells, phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the cell membrane, where it can be conjugated with annexin V. The cells were seeded in six-well plates and kept at 37°C for 24 hours. After incubation with the different solutions, they were gently detached and collected after a 10-minute incubation in 0.5 mM EDTA. Then, they were washed in PBS and suspended in 1 mL of PBS. An annexin V-FITC kit (Immunotech, Marseille, France) was used to assess annexin V-positive cells: 10 μ L of annexin V-FITC and 5 μ L of 7-aminoactinomycin D (7-AAD) were incubated for 10 minutes before flow cytometry analysis (Epics XL flow cytometer; Beckman Coulter). 7-AAD staining was used to identify cells that had lost membrane integrity and were thus classified as being necrotic rather than apoptotic.³⁵

Statistical Analysis

Results were obtained in fluorescence units and were expressed as a percentage of the control. Each drug concentration was tested in six wells, and each experiment was performed in triplicate. The mean values for each concentration were analyzed by one-way ANOVA followed by the Dunnett test^{36,37} (Sigma Stat 2.0; SSPS, Chicago, IL) and the level of significance was fixed at 0.05.

RESULTS

Cell Viability and Membrane Integrity Evaluation

No difference in cell viability was observed between ofloxacin without BAC (Oflo-BAC) and the control, after a 15-minute incubation (Fig. 1). In contrast, membrane integrity significantly decreased after a 15-minute incubation with ofloxacin containing BAC. The toxicity also appeared at each tested BAC concentration (0.0025%, 0.005%, and 0.01%) with significant loss of membrane integrity (−42%, −65%, and −58%, respectively; $P < 0.001$ compared with the control).

The alteration of membrane integrity was persistent (Fig. 2). After a 24-hour recovery period, the cytotoxic effect was further increased compared with the results in Fig. 1 for preserved ofloxacin and 0.0025% BAC.

ROS Production Assessment: DCFH-DA Test

DCFH-DA/neutral red ratio was calculated to take into account the number of living cells responsible for ROS synthesis (Fig.

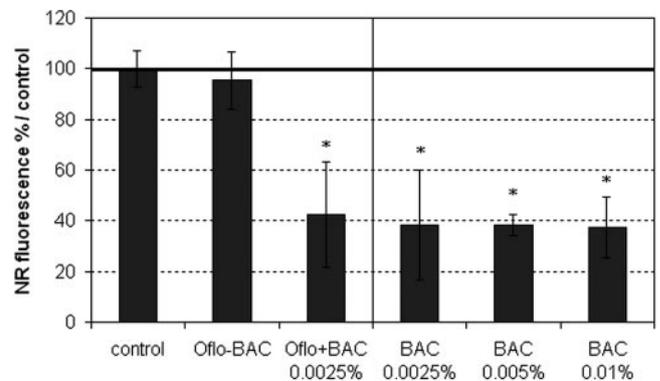


FIGURE 2. Membrane integrity evaluation by neutral red (NR) test after incubation with unpreserved or preserved ofloxacin and different concentrations of BAC. After 15 minutes of incubation followed by a 24-hour recovery period, there was a significant decrease in membrane integrity with preserved ofloxacin and BAC treatments, but not with unpreserved ofloxacin. * $P < 0.001$ compared with control.

3). Significant ROS production was observed with both unpreserved and preserved ofloxacin, but ofloxacin with BAC induced a higher production (+470% versus control) than without BAC (+310% versus control). BAC at 0.0025%, 0.005%, and 0.01% also provoked a nonlinear concentration-dependent increase in ROS production, 340%, 360%, and 530%, respectively, compared with the control.

O₂^{•−} Production Assessment: Dihydroethidium Test

Dihydroethidium (HE)/neutral red ratio was calculated. Significant O₂^{•−} production was observed for both preserved and unpreserved ofloxacin, but O₂^{•−} production with unpreserved ofloxacin was significantly less than with preserved ofloxacin (Fig. 4), 140% and 280%, respectively, compared with the control. BAC at 0.0025%, 0.005%, and 0.01% provoked a nonlinear concentration-dependent increase in O₂^{•−} production, 270%, 270%, and 350% respectively compared with the control.

Evaluation of P2X7 Cell Death Receptor Activation: YO-PRO-1 Test

In both ocular cell lines, no variation in YO-PRO-1 fluorescence signal was observed after ofloxacin without BAC incubation compared with the control (Fig. 5). Nevertheless, ofloxacin

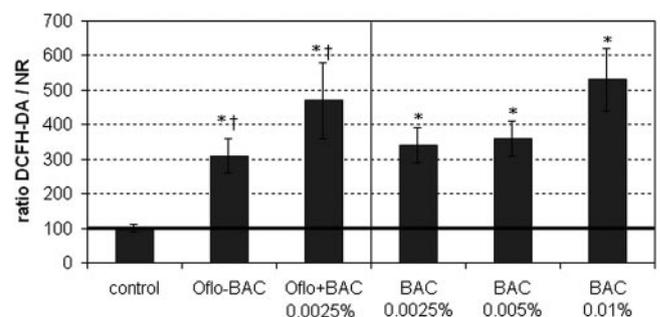


FIGURE 3. ROS production evaluation by DCFH-DA test after 15 minutes of incubation with unpreserved or preserved ofloxacin and different concentrations of BAC. There was a significant increase of ROS production with both unpreserved and preserved ofloxacin, but ofloxacin with BAC and the BAC solutions induced a much higher production. * $P < 0.001$ compared with the control; † $P < 0.001$ compared with each other.

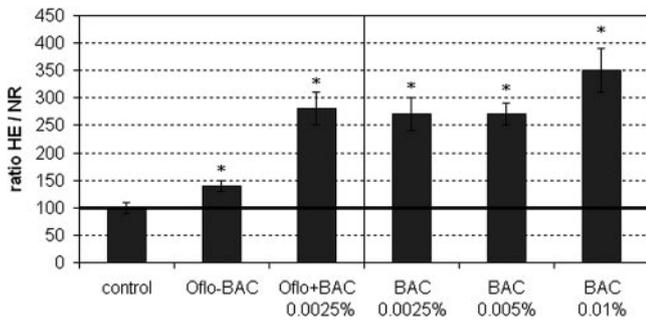


FIGURE 4. Superoxide anion ($O_2^{\cdot-}$) production evaluation with a dihydroethidium (HE) test after 15 minutes of incubation with unpreserved or preserved ofloxacin and different concentrations of BAC. There was a significant increase of ROS production with both unpreserved and preserved ofloxacin, but ofloxacin with BAC and the BAC solutions induced much higher production. * $P < 0.001$ compared with control.

with BAC produced a significant increase in YO-PRO-1 fluorescence signal, and this variation was not significantly different from that observed with 0.0025% BAC. At the highest BAC concentrations (0.005% and 0.01%), the YO-PRO-1 fluorescence signal greatly increased.

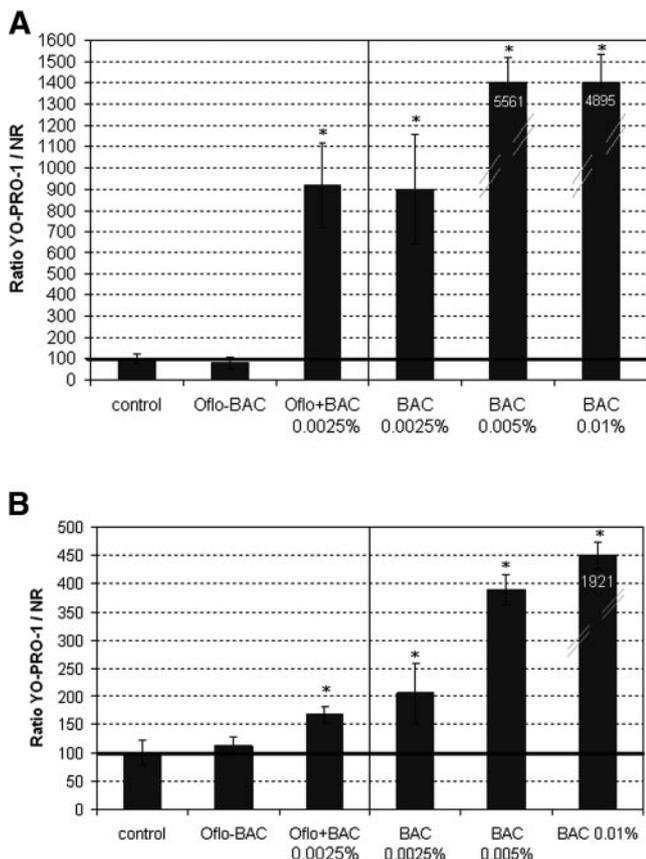


FIGURE 5. (A) Evaluation of P2X7 cell death receptor activation with YO-PRO-1 test after 15 minutes of incubation with unpreserved or preserved ofloxacin and different concentrations of BAC in a corneal cell line. There was a significant, concentration-dependent increase of YO-PRO-1 fluorescence with preserved ofloxacin and BAC solutions. * $P < 0.001$ compared with the control. (B) Evaluation of P2X7 cell death receptor activation with a YO-PRO-1 test after 15 minutes of incubation with unpreserved or preserved ofloxacin and different concentrations of BAC in a conjunctival cell line. * $P < 0.001$ compared with control.

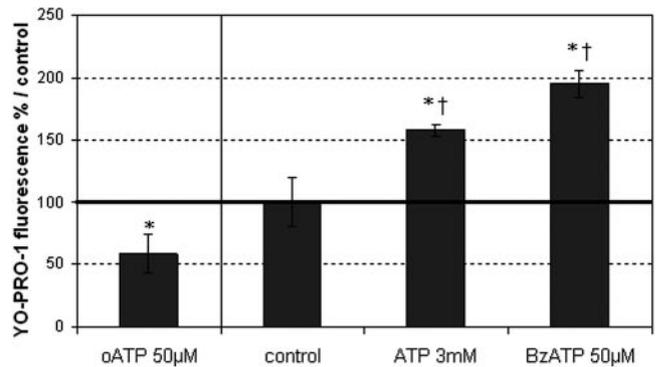


FIGURE 6. Evaluation of YO-PRO-1 specificity toward P2X7 pore opening. ATP and BzATP stimulated P2X7 pore opening whereas oATP inhibited it. * $P < 0.001$ compared with the control; † $P < 0.001$ compared with each other.

oATP induced a decrease in the YO-PRO-1 fluorescence signal, whereas ATP and BzATP both induced an increase in the fluorescence signal, with a higher increase for BzATP (Fig. 6).

Apoptosis: Chromatin Condensation Assessment—Hoechst 33342 Test

Cells incubated with ofloxacin without BAC showed the same intensity of fluorescence as the control cells (cells with no chromatin condensation; Fig. 7). Preserved ofloxacin and 0.0025% BAC induced a chromatin condensation specific to apoptosis, when compared with control cells. Moreover, cells incubated with different BAC concentrations showed chromatin condensation in a concentration-dependent manner.

Apoptosis: Phosphatidylserine Exposure Assessment—Annexin V Test

Cells incubated with ofloxacin alone showed the same intensity of fluorescence as did the control cells (Fig. 8). Both preserved ofloxacin and 0.0025% BAC induced an increase in annexin V-FITC fluorescence signal, which is specific to apoptosis.

DISCUSSION

BAC is used in pharmaceutical preparations for antimicrobial preservation because of its ability to alter bacterial membranes. It acts as a detergent, which can cause cellular damage by emulsification of the host lipids. Numerous clinical side effects of surfactant preservatives have been described, such as ocular irritation (photophobia, hyperemia, edema), punctate keratitis, gray corneal epithelial haze, pseudomembrane formation, and toxicity to the corneal epithelial cells.³⁸⁻⁴⁰

Fluoroquinolones promote great therapeutic progress due to their pharmacokinetic properties—oral administration, long half-life, and wide tissular diffusion—and their pharmacodynamic properties—wide antibacterial spectrum and bactericidal activity. The therapeutic choice of fluoroquinolones for treatment of external ocular infections depends on their local toxicity, because drugs are administered to an already injured ocular surface linked to keratitis or conjunctivitis.

Local toxicity may be caused by the preservative (BAC) or by the fluoroquinolone itself, motivating our investigation into the respective cytotoxic effect of BAC and/or fluoroquinolone on ocular surface cell lines.

The SIRC cell line is a well-known and established cell culture model that mimics corneal epithelium in pharmaco-

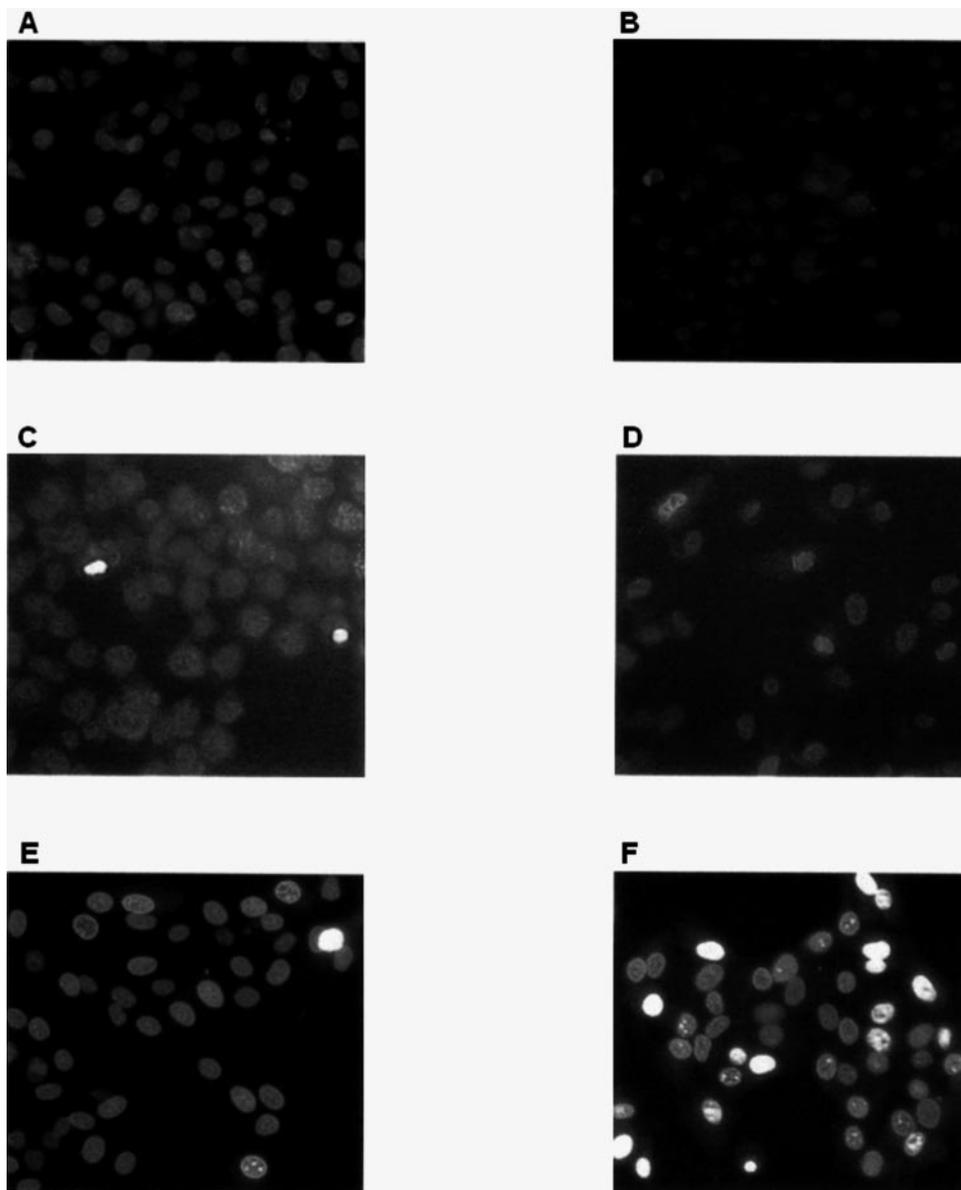


FIGURE 7. Evaluation of chromatin condensation with Hoechst 33342 test after 15 minutes of incubation with unpreserved (**B**), preserved ofloxacin (**C**) and different concentrations of BAC: (**D**) 0.0025%, (**E**) 0.005%, and (**F**) 0.01%. (**A**) Control culture. Preserved ofloxacin and different concentrations of BAC showed a characteristic concentration-dependent apoptotic chromatin condensation. Magnification, $\times 200$.

toxicological studies.^{41,42} The present results show that preserved ofloxacin induced a decrease in cell viability (necrosis) after 15 minutes of exposure, whereas unpreserved ofloxacin did not alter membrane integrity. After 0.0025% BAC exposure that corresponds to eye-drop BAC concentration, the same decrease in cell viability was observed, suggesting that BAC, but not ofloxacin itself, is responsible for cell death. This cytotoxic effect was persistent over 24 hours for BAC and preserved ofloxacin.

ROS evaluation showed a significant increase in ROS and O_2^- production. Different concentrations of BAC induced ROS production, but it remained at a lower level than for preserved ofloxacin. Unpreserved ofloxacin was responsible for a much lower production than preserved ofloxacin, and the results were still significant. Therefore, ROS production observed with preserved ofloxacin could be a result of a synergetic effect between ofloxacin itself and 0.0025% BAC.

Fluoroquinolones induce oxidative H_2O_2 production⁴³ controlled by the ocular detoxification system (glutathione, catalase, peroxidase). This system allows cells to resist H_2O_2 . However, cells are much more sensitive to O_2^- , and O_2^- production is known to cause apoptosis in the eye.^{44,45} O_2^-

could therefore play a role in the decrease of membrane integrity, these two parameters being correlated in our experiments. Moreover, O_2^- overproduction was associated with an increase in YO-PRO-1 fluorescence signal, and several studies have reported that P2X7 pores open in the early stages of apoptosis.⁴⁶

Due to the ultraviolet autofluorescence of fluoroquinolones, quantification by microplate cold light fluorometry with Hoechst 33342 was difficult; therefore a YO-PRO-1 test was assessed because this fluorogen is a visible fluorescent DNA stainer. The problem of interference between Hoechst 33342 and fluoroquinolone fluorescence was then avoided. Unpreserved ofloxacin and control cells expressed the same YO-PRO-1 fluorescence signal, whereas preserved ofloxacin showed the same fluorescence signal as 0.0025% BAC (no significant difference). An increase in YO-PRO-1 fluorescence signal associated with a decrease in neutral red fluorescence signal was observed for both preserved ofloxacin and 0.0025% BAC, leading to the conclusion that there is a BAC-induced apoptonecrosis. Because it has been shown that BAC incubated on primary rabbit corneal cell culture induces a decrease in the ATP/ADP ratio and an increase in $[Ca^{2+}]_i$, which are both

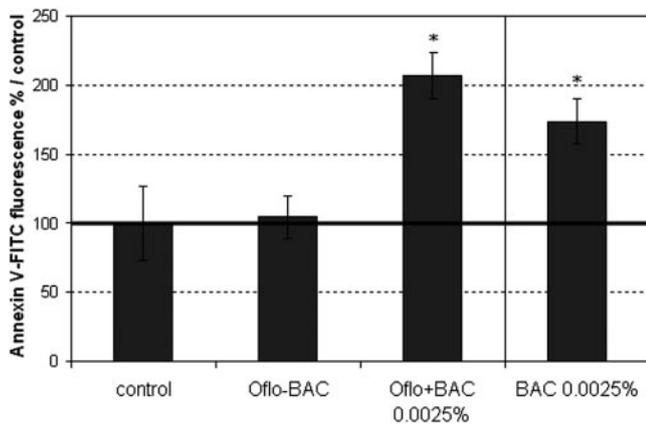


FIGURE 8. Evaluation of PS translocation by annexin V test after 15 minutes of incubation with unpreserved or preserved ofloxacin and BAC. Preserved ofloxacin and BAC at the same concentration both induced phosphatidylserine translocation. * $P < 0.001$ compared with control.

consequences of P2X7 pore opening leading to apoptosis,^{47,48} the implication of P2X7 cell death receptor activation by BAC appears highly plausible. Moreover, we observed different thresholds in YO-PRO-1 fluorescence signals in the corneal cell line; when BAC concentration was doubled from 0.0025% to 0.005%, fluorescence signal was increased more than fourfold, whereas when BAC concentration was doubled from 0.005% to 0.01%, the fluorescence signal did not significantly vary and finally when BAC concentration was increased from 0.01% to 0.1%, the fluorescence signal was increased twofold because of the number of necrotic cells (data not shown). These results strongly suggest the activation of a receptor: at lowest BAC concentrations, the P2X7 cell death receptor activation induces apoptosis, whereas at high BAC concentrations, the P2X7 cell death receptor acts as a cytolytic receptor (P2Z/P2X7 receptor) leading to cytolysis.⁴⁹ In addition, this could explain why we did not observe a linear concentration-dependent effect of BAC. There are different degrees of P2X7 receptor activation through different thresholds. Thresholds were also observed with the conjunctival cell line. The two tested cell lines did not stimulate the same level of P2X7 receptor activation. Corneal cells seem to be more sensitive than conjunctival cells since preserved ofloxacin induced a 900% increase in P2X7 receptor activation on corneal cells, whereas it induced a 170% increase in P2X7 receptor activation on conjunctival cells.

BAC induced P2X7 receptor stimulation through the formation of large pores, leading to membrane permeability disruption, with intra- and intercellular variations. BAC acts as a detergent that induces membrane emulsification, and so it was logical to observe high membrane injury after BAC incubation. This could justify the large standard deviations we observed with the neutral red and YO-PRO-1 tests, since they are both cell membrane permeability assays: neutral red dye evaluated cell viability through membrane integrity, and YO-PRO-1 dye evaluated apoptosis through the formation of membrane pores. In any event, data were significantly different from the control data ($P < 0.001$).

It is well known that the P2X7 receptor, which belongs to the purinoreceptor family, is specifically activated by ATP and BzATP, with higher sensitivity for BzATP.⁴⁹ In contrast, it is specifically inhibited by oATP.^{50,51} In our study, we confirmed these specific activations and inhibition of P2X7 receptor on two different models.

Finally, chromatin condensation was evaluated using Hoechst 33342 test with fluorescence microscopy. Hoechst

33342 uptake combined with propidium iodide to identify necrotic and late apoptotic cells is a well-known flow cytometry assay for apoptosis.⁵² Chromatin condensation induces differences in the spectral properties of Hoechst 33342, and apoptotic cells appear with an intense and nearly white fluorescent nuclear binding. It is currently used in cold light cytofluorometry but the quantification after fluoroquinolone incubation is difficult, because of the autofluorescence of fluoroquinolones. Apoptosis was confirmed for preserved ofloxacin as well as for different concentrations of BAC. In fact, the increase in Hoechst 33342 and annexin V-FITC fluorescence showed an increase in chromatin condensation and PS exposure, both resulting from the apoptotic phenomenon. In addition to the increase in chromatin condensation, preserved ofloxacin and BAC were responsible for a decrease in cell viability (neutral red test). A decrease in cell viability together with an increase in chromatin condensation reflects cellular death by apoptonecrosis. In previous studies BAC-induced apoptosis has been observed (at the same BAC concentrations and incubation times as in our tests) through the expression of the apoptotic marker Apo 2.7, an alteration of mitochondrial transmembrane potential and a decrease in glutathione, the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay and annexin V binding in different ocular cell models.^{8,10,53,54} A recent paper suggested that P2X7 receptors can be associated with pseudoapoptosis; meaning that if the stimulation of the receptor is brief (BzATP stimulation), the induced apoptotic events (plasma membrane PS flip, mitochondrial alterations, cytoskeletal disruption, and membrane blebbing) are reversible. BzATP was then found to induce pseudoapoptosis after short incubation times.⁵⁵ These new findings are interesting and contribute to a better understanding of particular P2X7 receptor-induced pathways but do not concern our present study, because our team specifically showed that BAC induced irreversible apoptosis (DNA fragmentation and cell cycle sub- G_1 peak tests) after a 10-minute incubation time followed by a 24-hour recovery period.⁸ Compared with BzATP, BAC induced irreversible apoptosis within 15 minutes.

In our study, we observed that BAC induced oxidative stress (reactive oxygen species and superoxide anion overproductions), apoptosis (chromatin condensation and phosphatidylserine translocation) and P2X7 cell death receptor activation. Our study does not allow us to make a direct link between these three elements but previous papers showed a correlation between superoxide anion overproduction and P2X7 receptor stimulation.¹³

In conclusion, fluoroquinolone eye-drop cytotoxicity seems to be mainly caused by the preservative. Our study highlights the important difference between cellular toxicity of BAC-preserved and preservative-free fluoroquinolone medications, which is in agreement with clinical and in vivo observations after fluoroquinolone topical ophthalmic formulation treatments. High stimulation of P2X7 cell death receptor activity (+900% to +5500%), which then acts as a P2Z/P2X7 cytolytic receptor,⁴⁹ could explain the corneal perforations observed after repeated treatment with fluoroquinolone in preservative.

Antibiotics such as fluoroquinolones do not need preservatives, as they already possess an antibacterial effect. Therefore, development of preservative-free solutions should be promoted in the future, especially as some papers have already reported the cytotoxic effects of BAC contained in artificial tear solutions and other topical ophthalmic medications.⁵⁶⁻⁵⁸ Our results therefore suggest that administration of preservative-free formulations to the eye would be safer, particularly in long-term antibiotic treatment and repeated treatments (more than eight times a day).

References

- Serdarevic ON. Role of fluoroquinolones in ophthalmology. *Int Ophthalmol Clin.* 1993;33:163-175.
- Cutarelli PE, Lass JH, Lazarus HM, Putman SC, Jacobs MR. Topical fluoroquinolones: antimicrobial activity and in vitro corneal epithelial toxicity. *Curr Eye Res.* 1991;10:557-563.
- Mallari P, Mccarty DJ, Daniell M, Taylor H. Increased incidence of corneal perforation after topical fluoroquinolone treatment for microbial keratitis. *Am J Ophthalmol.* 2001;131:131-133.
- Nelson JD, Siverman V, Lima PH, Beckman G. Corneal epithelial wound healing: a tissue culture assay on the effect of antibiotics. *Curr Eye Res.* 1990;9:277-285.
- Scuderi AC, Paladino GM, Marino C, Trombetta F. *In vitro* toxicity of netilmicin and ofloxacin on corneal epithelial cells. *Cornea.* 2003;22:468-472.
- Green K, Chapman JM, Cheeks L, Clayton RM, Wilson M, Zehir A. Detergent penetration into young and adult rabbit eyes: comparative pharmacokinetics. *J Toxicol Cut Ocul Toxicol.* 1987;6:89-107.
- Baudouin C, Pisella PJ, Fillacier K, et al. Ocular surface inflammatory changes induced by topical antiglaucoma drugs: human and animal studies. *Ophthalmology.* 1999;106:556-563.
- De Saint Jean M, Brignole F, Bringuier A, Bauchet A, Feldmann G, Baudouin C. Effects of benzalkonium chloride on growth and survival of Chang conjunctival cells. *Invest Ophthalmol Vis Sci.* 1999;40:619-630.
- Adams J, Wilcox MJ, Trousdale MD, Chien D, Shimizu RW. Morphologic and physiologic effects of artificial tear formulations on corneal epithelial derived cells. *Cornea.* 1992;11:234-241.
- Debbasch C, Brignole F, Pisella PJ, Warnet JM, Baudouin C. Quaternary ammoniums and other preservatives' contribution in oxidative stress and apoptosis on Chang conjunctival cells. *Invest Ophthalmol Vis Sci.* 2001;42:642-652.
- Le Feuvre R, Brough D, Rothwell N. Extracellular ATP and P2X7 receptors in neurodegeneration. *Eur J Pharmacol.* 2002;447:261-269.
- Gendron FP, Neary JT, Theiss PM, Sun GY, Gonzalez FA, Weisman GA. Mechanisms of P2X7 receptor-mediated ERK1/2 phosphorylation in human astrocytoma cells. *Am J Physiol.* 2003;284:C571-C581.
- Parvathenani LK, Tertysnikova S, Greco CR, Roberts SB, Robertson B, Posmantur R. P2X7 mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. *J Biol Chem.* 2003;278:13309-13317.
- Boucsein C, Zacharias R, Farber K, Pavlovic S, Hanisch UK, Kettenmann H. Purinergic receptors on microglial cells: functional expression in acute brain slices and modulation of microglial activation *in vitro.* *Eur J Neurosci.* 2003;17:2267-2276.
- Miras-Portugal MT, Diaz-Hernandez M, Giraldez LN, et al. P2X7 receptors in rat brain: presence in synaptic terminals and granule cells. *Neurochem Res.* 2003;28:1597-1605.
- Adinolfi E, Melchiorri L, Falzoni S, et al. P2X7 receptor expression in evolutive and indolent forms of chronic B lymphocytic leukemia. *Blood.* 2002;99:706-708.
- Labasi JM, Petrushova N, Donovan C, et al. Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol.* 2002;168:6436-6445.
- Budagian V, Bulanova E, Brovko L, et al. Signaling through P2X7 receptor in human T cells involves p56lck, MAP kinases, and transcription factors AP-1 and NF-kappa B. *J Biol Chem.* 2003;278:1549-1560.
- Dell'Antonio G, Quattrini A, Cin ED, Fulgenzi A, Ferrero ME. Relief of inflammatory pain in rats by local use of the selective P2X7 ATP receptor inhibitor, oxidized ATP. *Arthritis Rheum.* 2002;46:3378-3385.
- Wang CM, Chang YY, Sun SH. Activation of P2X7 purinoreceptor-stimulated TGF-beta 1 mRNA expression involves PKC/MAPK signalling pathway in a rat brain-derived type-2 astrocyte cell line, RBA-2. *Cell Signal.* 2003;15:1129-1137.
- Gudipaty L, Munetz J, Verhoef PA, Dubyak GR. Essential role for Ca²⁺ in regulation of IL-1 beta secretion by P2X7 nucleotide receptor in monocytes, macrophages, and HEK-293 cells. *Am J Physiol.* 2003;285:C286-C299.
- Colomar A, Marty V, Medina C, Combe C, Parnet P, Amedee T. Maturation and release of interleukin-1 beta by lipopolysaccharide-primed mouse Schwann cells require the stimulation of P2X7 receptors. *J Biol Chem.* 2003;278:30732-30740.
- Humphreys BD, Dubyak GR. Modulation of P2X7 nucleotide receptor expression by pro- and anti-inflammatory stimuli in THP-1 monocytes. *J Leukoc Biol.* 1998;64:265-273.
- Harada H, Chan CM, Loesch A, Unwin R, Burnstock G. Induction of proliferation and apoptotic cell death via P2Y and P2X receptors, respectively, in rat glomerular mesangial cells. *Kidney Int.* 2000;57:949-958.
- Von Albertini M, Palmethofer A, Kaczmarek E, et al. Extracellular ATP and ADP activate transcription factor NF-kappa B and induce endothelial cell apoptosis. *Biochem Biophys Res Commun.* 1998;248:822-829.
- Di Virgilio F. The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death. *Immunol Today.* 1995;16:524-528.
- Smart ML, Gu B, Panchal RG, et al. P2X7 receptor cell surface expression and cytolitic pore formation are regulated by a distal C-terminal region. *J Biol Chem.* 2003;278:8853-8860.
- Groschel-Stewart U, Bardini M, Robson T, Burnstock G. Localisation of P2X5 and P2X7 receptors by immunohistochemistry in rat stratified squamous epithelia. *Cell Tissue Res.* 1999;296:599-605.
- Kovoor TA, Kim AS, McCulley JP, et al. Evaluation of the corneal effects of topical ophthalmic fluoroquinolones using *in vivo* confocal microscopy. *Eye Contact Lens.* 2004;30:90-94.
- Rat P, Korwin-Zmilowska C, Warnet JM, Adolphe M. New *in vitro* fluorometric microtitration assays for toxicological screening of drugs. *Cell Biol Toxicol.* 1994;10:329-337.
- Balls M, Fentem JH. The use of basal cytotoxicity and target organ toxicity tests in hazard identification and risk assessment. *ATLA.* 1992;20:368-388.
- Borenfreud E, Puerner JA. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). *J Tissue Cult Methods.* 1984;9:7-9.
- Idziorek T, Estaquier J, De Bels F, Ameisen J-C. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J Immunol Methods.* 1995;185:249-258.
- Choucroun P, Gillet D, Dorange G, Sawicki B, Dewitte JD. Comet assay and early apoptosis. *Mutat Res.* 2001;478:89-96.
- Eckstein LA, Van Quill KR, Bui SK, Uusitalo MS, O'Brien JM. Cyclosporin A inhibits calcineurin/nuclear factor of activated T-cells signaling and induces apoptosis in retinoblastoma cells. *Invest Ophthalmol Vis Sci.* 2005;46:782-790.
- Dunnnett CW. A multiple comparison procedure for comparing several treatments with a control. *J Am Statist Assoc.* 1955;50:1096-1121.
- Dunnnett CW. New tables for multiple comparisons with a control. *Biometrics.* 1964;20:782-791.
- Gasset AR, Kaufman HE, Miller T. Cytotoxicity of ophthalmic preservatives. *Am J Ophthalmol.* 1974;78:98-105.
- Burstein NL. Preservative cytotoxic threshold for benzalkonium chloride and chlorhexidine digluconate in cat and rabbit corneas. *Invest Ophthalmol Vis Sci.* 1980;32:2259-2265.
- Gasset AR. Benzalkonium chloride toxicity to the human cornea. *Am J Ophthalmol.* 1977;84:169-171.
- Goskonda VR, Khan MA, Hutak CM, Reddy IK. Permeability characteristics of novel mydriatic agents using an *in vitro* cell culture model that utilizes SIRC rabbit corneal cells. *J Pharm Sci.* 1999;88:180-184.
- Goskonda VR, Hill RA, Khan MA, Reddy IK. Permeability of chemical delivery systems across rabbit corneal (SIRC) cell line and isolated corneas: a comparative study. *Pharm Dev Technol.* 2000;5:409-416.
- Pouzaud F, Bernard-Beaubois K, Thevenin M, Warnet JM, Hayem G, Rat P. *In vitro* discrimination of fluoroquinolones toxicity on tendon cells: involvement of Oxidative Stress. *J Pharmacol Exp Ther.* 2003;308:394-402.
- Debbasch C, De Saint-Jean M, Pisella PJ, Rat P, Warnet JM, Baudouin C. Quaternary ammonium cytotoxicity in a human conjunctival cell line. *J Fr Ophthalmol.* 1999;22:950-958.

45. Debbasch C, Pisella PJ, De Saint-Jean M, Rat P, Warnet JM, Baudouin C. Mitochondrial activity and glutathione injury in apoptosis induced by unpreserved and preserved beta-blockers on Chang conjunctival cells. *Invest Ophthalmol Vis Sci.* 2001;42:2525-2533.
46. Virginio C, MacKenzie A, North RA, Surprenant A. Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X7 receptor. *J Physiol.* 1999;519:335-346.
47. Grant RL, Acosta D. Prolonged adverse effects of benzalkonium chloride and sodium dodecylsulfate in a primary culture system of rabbit corneal epithelial cells. *Fund Appl Toxicol.* 1996;33:71-82.
48. Schulze-Lohoff E, Hugo C, Rost S, et al. Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via P2Z/P2X7 receptors. *Am J Physiol.* 1998;275:962-971.
49. Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science.* 1996;272:735-738.
50. Kim M, Spelta V, Sim J, North RA, Surprenant A. Differential assembly of rat purinergic P2X7 receptor in immune cells of the brain and periphery. *J Biol Chem.* 2001;276:23262-23267.
51. Virginio C, Church D, North RA, Surprenant A. Effects of divalent cations, protons and calmidazolium at the rat P2X7 receptor. *Neuropharmacology.* 1997;36:1285-1294.
52. Belloc F, Dumain P, Boisseau MR, et al. A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. *Cytometry.* 1994;17:59-65.
53. Hamard P, Blondin C, Debbasch C, Warnet JM, Baudouin C, Brignole F. *In vitro* effects of preserved and unpreserved antiglaucoma drugs on apoptotic marker expression by human trabecular cells. *Graefes Arch Clin Exp Ophthalmol.* 2003;41:1037-1043.
54. Dogan AS, Orhan M, Soylemezoglu F, Irkeç M, Bozkurt B. Effects of topical antiglaucoma drugs on apoptosis rates of conjunctival epithelial cells in glaucoma patients. *Clin Exp Ophthalmol.* 2004;32:62-66.
55. MacKenzie AB, Young MT, Adinolfi E, Surprenant A. Pseudoapoptosis induced by brief activation of ATP-gated P2X7 receptors. *J Biol Chem.* 2005;280:33968-33976.
56. De Saint Jean M, Debbasch C, Brignole F, Rat P, Warnet JM, Baudouin C. Toxicity of preserved and unpreserved antiglaucoma topical drugs in an *in vitro* model of conjunctival cells. *Curr Eye Res.* 2000;20:85-94.
57. Lazarus HN, Imperia PS, Botti RE. An *in vitro* method which assesses corneal epithelial toxicity due to antineoplastic, preservative and antimicrobial agent. *Lens Eye Toxic Res.* 1989;6:59-85.
58. Tripathi BJ, Tripathi RC. Cytotoxic effect of benzalkonium chloride and chlorobutanol on human corneal epithelial cells *in vitro*. *Lens Eye Toxic Res.* 1989;6:395-403.