Preservatives in eyedrops: The good, the bad and the ugly

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A B S T R A C T

There is a large body of evidence from experimental and clinical studies showing that the long-term use of topical drugs may induce ocular surface changes, causing ocular discomfort, tear film instability, conjunctival inflammation, subconjunctival fibrosis, epithelial apoptosis, corneal surface impairment, and the potential risk of failure for further glaucoma surgery. Subclinical inflammation has also been described in patients receiving antiglaucoma treatments for long periods of time. However, the mechanisms involved, i.e., allergic, toxic, or inflammatory, as well as the respective roles of the active compound and the preservative in inducing the toxic and/or proinflammatory effects of ophthalmic solutions, is still being debated. The most frequently used preservative, benzalkonium chloride (BAK), has consistently demonstrated its toxic effects in laboratory, experimental, and clinical studies. As a quaternary ammonium, this compound has been shown to cause tear film instability, loss of goblet cells, conjunctival squamous metaplasia and apoptosis, disruption of the corneal epithelium barrier, and damage to deeper ocular tissues. The mechanisms causing these effects have not been fully elucidated, although the involvement of immunoinflammatory reactions with the release of proinflammatory cytokines, apoptosis, oxidative stress, as well as direct interactions with the lipid components of the tear film and cell membranes have been well established. Preservative-induced adverse effects are therefore far from being restricted to only allergic reactions, and side effects are often very difficult to identify because they mostly occur in a delayed or poorly specific manner. Care should therefore be taken to avoid the long-term use of preservatives, otherwise a less toxic alternative to BAK should be developed, as this weakly allergenic but highly toxic compound exerts dose- and time-dependent effects. On the basis of all these experimental and clinical reports, it would be advisable to use benzalkonium-free solutions whenever possible, especially in patients with the greatest exposure to high doses or prolonged treatments, in those suffering from preexisting or concomitant ocular surface diseases, and those experiencing side effects related to the ocular surface. Indeed, mild symptoms should not be underestimated, neglected, or denied, because they may very well be the apparent manifestations of more severe, potentially threatening subclinical reactions that may later cause major concerns.

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* Financial disclosure: Christophe Baudouin is consultant for or has received research grants from: Alcon, Allergan, MSD, Pfizer, Santen and Théa. The other authors have benefitted from research grants from the same companies.
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doi:10.1016/j.preteyeres.2010.03.001
1. Introduction

In the recent past, a large body of evidence from experimental and clinical studies has accumulated showing that the long-term use of topical drugs may induce major and frequent ocular surface impairment, such as dry eye, meibomian gland dysfunction, or chronic allergy, and are very frequently treated with two or more topical ophthalmic formulations. These findings explain that clinical practice apparently often does not fit the safety profiles of drugs tested in clinical trials for short periods of time, alone, and only in patients without active ocular surface disorders.

The need for sterility in multidose eye drops requires the inclusion of an antimicrobial preservative in these solutions, most frequently the quaternary ammonium benzalkonium chloride (BAK). In some patients — on long-term treatments — allergic or inflammatory reactions are experienced, including redness, stinging, burning, irritation, eye dryness, or less frequently, conjunctivitis or corneal damage. Many studies indicate a direct correlation between the presence of preservatives and the symptoms experienced during antiglaucoma therapy. In a recent large-scale European observational study (Jaenen et al., 2007), involving 9658 patients, all recorded symptoms and signs were significantly more frequent in patients taking preserved medications compared with those taking preservative-free formulations. Most effects observed in glaucoma patients are therefore more likely to be due to the preservative than the active ingredients, since toxicity of the preservatives has been well documented experimentally, as has the harmlessness of most active compounds when unpreserved. BAK toxicity for eye structures has been reported since the 1940s (Swan, 1944) and many studies in experimental or cell models have consistently and reliably shown its toxic effects (Baudouin, 2008).

Conversely, BAK is a hapten causing relatively few short-term allergic reactions and appears in clinical trials as an effective and safe agent, especially when compared to mercury derivatives or chlorhexidine, formerly used in ophthalmic preparations. Nevertheless, given that the main characteristic of a toxic compound is that it exerts time- and dose-dependent effects, these effects are most likely to occur late, with poor specificity in its manifestations, when delayed, mild, or interacting with other ocular disorders. Moreover, in most cases, the severity of glaucoma as a sight-threatening condition could be underestimated.
disease makes dry eye sensation or chronic ocular irritation a comparatively very mild caveat regarding the benefit of glaucoma treatment. However, ocular surface manifestations deeply impact the quality of life and may influence patient compliance, in addition to a variety of long-term consequences for the eye. This review is aimed at reviewing the long-term ocular surface side effects of topical treatments, focusing on BAK toxicity and its potential harmfulness in ophthalmic preparations.

2. Preservatives in ophthalmic preparations

2.1. Regulatory aspects

The Pharmacopoeia recommends that eye drops must contain an antimicrobial agent (preservative) to avoid or to limit microbial proliferation after opening the bottle, which could induce a risk of potentially severe eye infection as well as the alteration of the formulation. Eye drops are contaminated essentially by the hands when handling the bottle or by contact of the tip touching the eyelids, lashes, conjunctiva, or tears. There is also a risk of cross-transmission when the same eye drops are shared by several patients, especially in a hospital environment or within the same family. Moreover, this type of incident in the 1960s in a Birmingham hospital triggered the British authorities to require the industry to develop the first unit-doses and to limit the duration of use after opening (cited in Chibret, 1997). As disposable single-dose units are less cost-effective than multidose bottles, the greatest progress has been made in the field of preservative development since the 1960s. However, the regulations requiring addition of an antimicrobial agent in any multidose ophthalmic preparation appeared only in the 1970s. Preservatives used in ophthalmic preparations belong to a variety of chemical families, including mercury derivatives, alcohols, parabens, EDTA, and chlorhexidine, but quaternary ammonium compounds, due to their low allergenic effects and apparently good safety profiles, have progressively become the major preservative of today’s Pharmacopoeia. The US Pharmacopoeia bases the efficacy of preservatives on the Preservative Effectiveness Test (PET) that consists of inoculation with 1x10^6 colony forming units (cfu)/mL at Day 0 with various organisms, namely bacteria (Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli) and fungi (Aspergillus niger and Candida albicans). The regulatory requirements are: 1.0-log reduction by day 7, 3.0-log reduction by day 14, no increase in survivors at Day 14, no increase in survivors from Days 14–28, and no increase in survivors for the fungi from Day 0 to Day 28 (Rosenthal et al., 2006). Indeed, such effectiveness on microorganisms through cytotoxic effects cannot be achieved without a minimal amount of toxicity to tissues where preservatives are applied. Authorities more and more concerned by such toxicological issues nowadays tend to favor the development by the industrials of new preservatives of preservative-free alternatives. In a recent statement, the EMEA (European Medicines Agency), thus addressed the interest of avoiding preservatives in “patients who do not tolerate eyedrops with preservatives” and those with long term treatment, of using “concentration at the minimum level consistent with satisfactory antimicrobial function in each individual preparation”, of promoting “new ophthalmic preparations without any mercury-containing preservatives”, although did not give a general recommendation not to use preservatives in eye drops (EMEA statement, 2009).

2.2. Benzalkonium chloride, the foremost modern preservative

Today, the most commonly used preservative in ophthalmic preparations is benzalkonium chloride. BAK is a nitrogenous cationic surface-acting agent belonging to the quaternary ammonium group. It has three main categories of use: as a biocide, a cationic surfactant, and a phase transfer agent in the chemical industry. Quaternary ammoniums are bipolar compounds, which are highly hydrosoluble and have surfactant properties. They act mainly via their detergent properties, which vary in strength and dissolve the bacterial walls and membranes, and destroy the semipermeable cytoplasmic layer. Their bactericidal activity is rapid and is greatest at 37°C in an alkaline medium. The spectrum of activity is mainly focused on Gram + bacteria (Staphylococcus) even at very low concentrations. Activity against Gram – bacteria (P. aeruginosa) is increased when it is combined with EDTA 0.1%. The quaternary ammoniums are also excellent fungicides and are particularly active against C. albicans and Aspergillus fumigatus. Finally, these compounds are potent spermiocides, and as well as being used in ophthalmic preparations, they are used in a wide range of commonly used products (soaps, cosmetics, cleaning products, disinfectants, etc.).

BAK is a mixture of alkylbenzyldimethylammonium chloride, whose general formula (Fig. 1) is: CnH2n+1N(CH3)2RCl, R representing the alkyl radicals from C8 to C18. BAK-C12 is called benzododecinium chloride, BAK-C14, myristalkonium chloride, and BAK-C16, cetalkonium chloride. It is commonly used at concentrations ranging between 0.004 and 0.025%. Several investigations using animal models suggested the existence of links between BAK and cytotoxic effects on several components of the eye. The key studies are outlined below.

3. Is BAK an appropriate compound?

3.1. Efficacy as a preservative

BAK was mainly used for its apparently good safety/efficacy profile. This compound is weakly allergenic and has a high rate of antimicrobial properties. In a study illustrating the potent antimicrobial properties of BAK, Charnock showed that among five preservatives, benzalkonium chloride/EDTA, parabens, chlorobutanol, silver chloride complex, and the Purite-stabilized oxychlo-complex, only BAK/EDTA satisfied the major criteria for antimicrobial preservation for all the test microorganisms. Moreover, it was the only one tested to inhibit S. aureus (Charnock, 2006). Indeed, BAK efficiently destroys the cell membranes of microorganisms and seems to have a good safety profile, even though it is almost impossible for a chemical to discriminate membranes of pathogens from those of normal eye cells.

Conversely, nonpreserved eyedrops could enhance the risk of contamination. Preservative-free artificial tears in reclosable containers were shown to be at risk of contamination after multiple uses over 10 h (Kim et al., 2008). The risk was higher in older patients and with inappropriate finger manipulation, which may be the hallmark of patients with chronic eye diseases, namely glaucoma or dry eye disease. However, it should be noted that even in the worst conditions of repeated handling of unpreserved eyedrops, only 2% of bottles appeared to be contaminated. Moreover, in a multiple-use setting, bottles preserved with BAK achieved a 34.8% rate of contamination after 15 days, thus demonstrating the very relative

![Chemical formula of Benzalkonium chloride (BAK).](image-url)
security offered by the preservative (Tasli and Cosar, 2001). Moreover, some strains of P. aeruginosa have been found to be resistant to BAK and can be grown in concentrated BAK solutions. The acquired resistance could be eliminated by the presence of EDTA in the solution, which may also act as an enhancer of corneal permeation (Pawar and Majumdar, 2006), but whose toxicity, especially when associated with BAK, has not been fully addressed.

### 3.2. Enhancement of penetration of active compounds

One major argument that is proposed to maintain the use of quaternary ammoniums in eyedrop formulations is that BAK reportedly enhances the penetration of the drug into the anterior chamber, through disruption of the hydrophobic barrier of the corneal epithelium. This property may result in a better concentration of the active compound and better stimulation/inhibition of the target receptors, so that better efficacy should be expected. The prostaglandin analog tafluprost was therefore tested in a pharmacokinetic study in rabbits, but it showed no difference in terms of drug concentrations in the aqueous humor between the preserved and unpreserved formulations (Pellinen and Lokkila, 2009). Conversely, topical cyclosporine A penetration into the cornea was found to be enhanced by a vehicle containing BAK, although almost no differences were observed in the iris/ciliary body (Venkate et al., 2008). BAK, especially when added to other enhancers such as chitosan or EDTA, was also shown to increase transcorneal permeation of acyclovir (Majumdar et al., 2008) and various antibacterials (Rathore and Majumdar, 2006).

Although major pharmacokinetic differences most likely exist between the large variety of currently available compounds and formulations (hydrophilic vs. lipophilic drugs, prodrugs vs. active compounds, solutions vs. suspension or emulsions, etc.), the pharmacokinetic characteristics of an eyedrop do not necessarily correlate its effectiveness, e.g., intraocular pressure (IOP) reduction. Indeed, besides the fact that enhancing penetration of a drug through major histological changes in the corneal barrier is actually a toxicity-related side effect (de Jong et al., 1994), several studies have addressed the question in terms of efficacy, showing the overall equivalence between preserved and unpreserved antiglaucomatous formulations at least for the tested compounds.

### 3.3. Absence of evidence of better IOP control in most BAK-containing eyedrops

The question of a possible difference in IOP reduction between BAK-containing and unpreserved antiglaucomatous drugs or BAK-free formulations was first addressed with beta-blockers, a family of hydrophilic compounds most susceptible to be influenced by a decreased penetration of the active compound into the anterior chamber. Although theoretically beta-blocker penetration should be reduced in the absence of BAK, this was not demonstrated for a topical betaxolol suspension (Denis et al., 1993) or carteolol: a comparative study of 2% carteolol with and without preservative also confirmed similar IOP reduction by the two formulations (Baudouin and de Lunardo, 1998). More recently, a study compared nonpreserved versus preserved timolol gel 0.1% and demonstrated that both formulations resulted in a non-significantly different reduction of IOP (Easty et al., 2006).

Concerning prostaglandin analogs, which are lipophilic compounds, similar results have been published. Two recent clinical trials compared the pharmacokinetic and pharmacodynamic profiles between preserved and preservative-free formulations of the prostaglandin analog tafluprost. There were no statistically significant differences between formulations with regards to pharmacokinetic parameters and systemic bioavailability (Uusitalo et al., 2008). There were no differences in IOP between preserved and preservative-free formulations in the two studies. The overall IOP reduction difference between preservative-free and preserved formulations was within ±0.46-mmHg (Hamacher et al., 2008).

In addition, several studies compared Travoprost with and without BAK, using a multidose preparation preserved with Sofzia6, an ionic buffered preservative system. The two showed no significant differences in terms of IOP control: in the first one (Lewis et al., 2007), at all time points the differences in IOPs measured during a 3-month study remained within a range of ±0.8 mmHg. The second one also showed no difference between both formulations even 60 h after the last administration of the prostaglandin analog (Gross et al., 2008). Likewise, the fixed combination of timolol and dorzolamide (Cosopt2, Merck, Sharpe & Dohme, NV) showed the equivalent efficacy on IOP control with preserved and preservative-free preparations (Lalovitz et al., 1999).

### 3.4. Pharmacokinetic data on BAK

Conversely, does BAK enter the eye or infiltrate ocular structures? This question is important regarding a potentially harmful compound that may cause very low toxic changes in the short term but is often used for the rest of the patient’s life and may accumulate in ocular tissues. To our knowledge, only one study has reported BAK pharmacokinetic data after instillation. BAK is retained in tissues and can be found 168 h after a single 30-µl drop of 0.01% BAK in rabbits. The half-life of BAK from corneal and conjunctival tissues in the epithelium is 20 h and the half-life in conjunctival deeper structures is 11 h (Champeau and Edelhauser, 1986). This is important to consider because tear washout may considerably reduce tear concentration of toxins, including BAK (Friedlaender et al., 2006), whereas deeper structures may be impregnated, especially after repeated use of several preservative-containing eyedrops over the long term.

### 3.5. Randomized clinical trials and the real world

Medical treatment is predominantly used as first-line therapy and the large majority of patients with chronic glaucoma are medically treated. Therefore medical treatment is administered over the long term and most of patients receive several decades of treatment, if not for most of their lifetime, as even those undergoing surgery often require further adjunctive medical therapy. Eyedrops were developed before being approved after thorough methodological evaluations using randomized clinical trials (RCTs). Based on data from RCTs, the tolerance of glaucoma treatments seems satisfactory, with only a few patients withdrawn due to local intolerance or allergy. Although prolonged clinical trials are seldom, medical antiglaucoma treatments therefore appear not harmful even if they will most likely be used for much longer periods of time (Alm et al., 1997; Cohen et al., 2004; Fellman et al., 2002).

Although RCTs usually demonstrated good safety profiles for newly developed eyedrops, it should be recognized that there are several major differences between clinical trials and real-life use of antiglaucoma therapy. First, clinical trials are usually of short duration (6 months to rarely more than 1 year). The time-dependent effects of toxic compounds may require years or decades to develop at a clinically identifiable level and may therefore be largely underestimated in shorter duration RCTs. Secondly, most often patients only receive one test or reference therapy and possible interactions or additive toxic effects of various compounds are not addressed. In addition, for ethical reasons patients with known hypersensitivity to the active molecule or the preservative, and patients who have active concomitant diseases, in particular those with severe dry eye, blepharitis or chronic allergy, are not
including in such trials, as these pathologies may strongly interfere with ocular tolerance to eyedrops, thus creating a selection bias, potentially underestimating the real-life situation. However, the prevalence of dry eyes in patients over 65 varies between 15 and 34% (Dry Eye Workshop, 2007b). Therefore, an ocular surface disease may be enhanced by the drug(s) and/or preservatives, and conversely may reduce the resistance of the cornea and conjunctiva to the toxic or irritant compounds.

Finally, in clinical practice, glaucoma very often evolves and requires additional treatments over time, a large proportion of patients thus receiving multiple therapies concomitantly. The Ocular Hypertension Treatment Study demonstrated that approximately 40% of patients initially diagnosed with ocular hypertension were using two drugs after 5 years, 9% three or more, with even higher rates in African-American patients (Kass et al., 2002). Multitherapy is likely to increase the incidence of adverse events, by additivity of side effects of each individual component, or possible between-drug interactions. This is particularly relevant for the preservative that may accumulate and reach high tissue levels when instilled four or five times a day. Indeed, Osborne et al. showed that the allergic effects of brimonidine might increase the propensity for multiple allergic reactions to subsequently used preparations (Osborne et al., 2005). This illustrates the role of between-drug interactions, both concomitantly and consecutively, and the likelihood of discrepancies between the clinical trial setting and clinical practice, when patients experiencing concomitant or preexisting ocular surface involvement are treated with several drugs for decades. This may indicate that the real world clearly trumps the reassuring results of RCTs and their low numbers of patients who are withdrawn from or intolerant to treatment. This must be taken into consideration and emphasizes the discrepancy between two major principles: evidence-based medicine and the precautionary principle. The former bases any assumption on RCTs, whereas, according to the latter, any doubtful compound, even if toxicity occurs only in a minority of patients, should be eliminated or alternatives developed.

4. The ocular surface of glaucomatous patients

4.1. Allergic reactions

The symptoms of conjunctival allergy (congestion, tearing, photophobia, burning and stinging sensations) induced by the instillation of preserved eyedrops are various. Simple conjunctival congestion or papillary conjunctivitis may be observed with or without eczema, the worst reaction being patent giant papillary conjunctivitis. In certain cases, a severe type IV allergic reaction may develop. Allergic manifestations are often spectacular (Fig. 2), occurring a few days after treatment initiation, and recover rapidly when treatment is stopped. In clinical trials, the overall rate of withdrawal is relatively low, a few percent of the patients recruited. In a meta-analysis of 28 randomized clinical trials, the overall rate of withdrawal was ranging from 0 to 16%, the majority of RCT having a rate <8% (van der Valk et al., 2005). However, delayed allergic reactions may occur, often mimicking blepharitis with low-grade inflammation (Fig. 3). In this condition, the relationship between the eyedrops and ocular inflammation is often difficult to assess, especially when the treatment is mandatory for a severe sight-threatening condition.

4.2. Dry eye symptoms

In contrast with data obtained from prospective clinical trials, the real world shows a much larger proportion of patients suffering from symptoms and signs related to the ocular surface, as assessed by several observational surveys. Some of them included such a high number of patients that their clinical value cannot be denied. An epidemiological survey was conducted in 2002 in 4107 glaucoma patients to assess the rate of ocular symptoms and conjunctival, corneal, and palpebral signs in a routine clinical practice (Pisella et al., 2002). Prevalence of symptoms was found to be very high in patients using preserved drops, with 43% discomfort upon instillation, 40% burning or stinging sensation, 31% foreign body sensation, 23% dry eyes, 21% tearing, and 18% itchy eyelids. Frequency of signs and symptoms increased with the number of preserved eye drops used and was significantly lower for all criteria in a group of patients treated with unpreserved betablockers. These results were found to be quite similar in several other European countries when the same study was conducted in Italy, Belgium, and Portugal. Pooled data from 9,658 patients demonstrated that the incidence of ocular symptoms ranged between 30 and 50% of patients (Fig. 4) (Jaenen et al., 2007). Likewise, a total of 20,506 patients from 900 centers across Germany were included in an observational study on dry eye prevalence in glaucoma. The first 30 consecutive glaucoma patients at each center were recruited. According to the registry data, more women develop dry eye and glaucoma than men (56.9 vs. 45.7%). Dry eye occurred more
frequently in more severe glaucoma cases, when three or more antiglaucoma drugs were used, and increased with the duration of glaucoma disease (Erb et al., 2008).

Similarly, in the United States, a cross-sectional study evaluated the impact of ocular surface in glaucoma management in 101 patients with open-angle glaucoma or ocular hypertension. Using the Ocular Surface Disease Index for measuring symptoms of dry eye, 59% of patients reported symptoms in at least one eye, qualified as severe in 27% of patients. Schirmer testing showed 61% of patients with decreased tear production in at least one eye, 35% being severely affected. Corneal and conjunctival lissamine green staining showed positive results in 22% of cases. Tear break-up time was decreased in 78% of patients (65% qualified as severe) (Leung et al., 2008).

More recently, a new observational survey confirmed the high prevalence of dry eye in glaucoma patients with a clear relationship with the number of eyedrops: 39% and 43% of dry eye with two and three eyedrops, respectively, whereas dry eye was only found in 11% of patients receiving only one eyedrop. Based on a score of ocular surface symptoms, severe dry eye was found in 8.7% and 15% of patients with two and three eyedrops, respectively (Rossi et al., 2009).

The last two studies concluded that ocular surface symptoms had a substantial impact on the patients’ quality of life, as is well known in dry eye disease irrespective of the cause (Dry Eye WorkShop, 2007b). The impact on compliance is very likely, although more difficult to assess. In an observational study conducted in 204 patients, the high rate of symptoms, with 25.4% experiencing burning sensation, 20.8% blurred vision, and 20.2% tearing, led to poor patient satisfaction and reduced adherence. Dissatisfied patients also visited their ophthalmologists more frequently (Nordmann et al., 2003). In a survey performed in the US between 2001 and 2004, based on the refilling rate of initial therapy in 300 patients, adverse effects were found the second most common reasons noted by physicians for switching medications after lack of efficacy (19% vs. 43%, respectively) (Zimmerman et al., 2009).

4.3. Subconjunctival fibrosis and pseudopemphigoid

The development of subconjunctival fibrosis has been reported in patients treated with antiglaucoma medications for a long period of time, most likely resulting from an increase in fibroblast density in the subepithelial substantia propria, related to an increase in inflammatory cells (Baudouin et al., 1999; Broadway et al., 1994a; Sherwood et al., 1989). The use of long-term antiglaucoma medications has been shown to cause conjunctival foreshortening and shrinkage, which may be associated with an ocular pemphigoid-like condition or evolve into severe scarring conjunctivitis with definitive corneal opacities (Schwab et al., 1992). In a series of 145 patients presenting a pseudopemphigoid, Thorne et al. showed that exposure to antiglaucoma eyedrops was the primary cause of pseudopemphigoid. Almost all the cases reported (97.4%) involved an association of antiglaucoma medications (Thorne et al., 2004).

4.4. Increased rate of glaucoma surgery failure

There have been several reports of long-term topical combination therapy as a significant risk factor for glaucoma surgery failure (Lavin et al., 1990; Broadway et al., 1994b). The success rate of trabeculectomy was thus found to be negatively influenced by long-term topical antiglaucoma therapy (Lavin et al., 1990). Broadway and colleagues further studied the effect of different long-term antiglaucoma treatments on the results of glaucoma filtration surgery and found a significant relationship between the risk of failure of filtering surgery and the number of drugs used and duration of treatment (Broadway et al., 1994b). The failure of glaucoma surgery in most cases originates from a fibroblastic conjunctival response at the bleb level in the early postoperative months. An excessive wound healing process characterized by inflammation and fibroblast proliferation and extracellular matrix deposition results in blockade of aqueous outflow in the subconjunctival space. Increased postoperative fibrosis may be enhanced in populations of patients or conditions known to be at higher risk of surgical failure, but several reports have pointed out the role played by preoperative inflammation of the conjunctiva caused by antiglaucomatous drugs used over the long term (Broadway and Chang, 2001).

Long-term therapy induces an inflammation of the conjunctiva with consequently an increased postoperative fibrosis. Therefore, the use of topical antiglaucoma drugs can be considered as an important risk factor for failure of filtration surgery and whenever possible, improvement of the ocular surface and reduction of inflammation prior to surgery should be considered (Broadway and Chang, 2001). Although these results were collected at a time when new powerful drugs had not yet been developed, more than 50% of glaucomatous patients require two or more drugs and are still operated on after prolonged medical treatment with multiple drugs. Filtration surgery success rates do not appear to be improved in the recent years (Gedde et al., 2009).

4.5. Histopathological data

As clinical data and observations often lack specificity, numerous reports have investigated the ocular surface of glaucomatous patients by means of histopathological techniques. They clearly demonstrated that, even without evident symptoms or clinical manifestations, inflammation is abnormally observed in the conjunctival epithelium and substantia propria. In most cases, conjunctival biopsies have been taken at the time of glaucoma surgery and examined using immunohistological techniques. Infiltration of immunocompetent cells in the conjunctiva has therefore been consistently reported. Sherwood et al. compared two groups of patients, one with primary glaucoma surgery, the other one after long-term eyedrop therapy (Sherwood et al., 1989). A significant increase in the number of macrophages, lymphocytes, mast cells, and fibroblasts in the conjunctiva and the Tenon capsule
and a significant decrease in the number of epithelial goblet cells were observed in the latter group. In another study, conducted on 124 conjunctival biopsy specimens from patients undergoing filtration surgery, Broadway et al. found that administration of topical medication, irrespective of type, for 3 years or more induced a significant degree of subclinical inflammation in the conjunctiva (Broadway et al., 1994a). Associations of two or more medications thus induced a significant decrease in goblet cells, an increase in pale cells, macrophages, and lymphocytes within the epithelium, and an increase in fibroblasts, macrophages, mast cells, and lymphocytes in the substantia propria. In addition, administration of one topical medication for more than 3 years was found to be associated with similar inflammatory and fibroblast infiltration. These data were directly correlated to risk of surgical failure (Broadway et al., 1994b). Similarly, in medium- and long-term therapy patients, Nuzzi et al. confirmed significant increases in the thickness and number of epithelial cell layers, in the fibroblast density in both subepithelial and deep connective tissue, and a more compact connective tissue, richer in collagen fibers arranged in whips, with some inflammatory elements and increased expression of inflammatory markers (HLA-DR, CD1a, CD4, CD8, IL-2, and C3b) (Nuzzi et al., 1995).

Our group also conducted an immunohistological study in conjunctival biopsies and trabecular meshwork specimens from patients undergoing glaucoma surgery after various durations and number of medical treatments. Immunohistochemical analyses revealed that samples from patients who were receiving treatments had significantly greater expression of fibroblastic and inflammatory markers compared with untreated participants. Furthermore, patients who received multiple therapies had greater expression of markers compared with those who were on mono-therapy (Baudouin et al., 1999). Apoptotic rates in the conjunctival epithelium were also found to be increased in glaucoma patients treated chronically compared to normal eyes, independent of drug family, which could again raise the possible if not likely role played by the preservative in epithelial toxicity (Dogan et al., 2004).

Conversely, the most common sign related to prostaglandin analogs, namely hyperemia, was histologically confirmed as resulting from vascular congestion (Leal et al., 2004) and was not associated with inflammatory changes when examining specimens receiving bimatoprost eye drops, a preparation that frequently causes hyperemia but contains low doses of BAK, i.e., 0.005%. However, little information is available on the conjunctival changes involved in patients receiving monotherapy of prostaglandin analogs. In conjunctival specimens from 20 patients treated with either latanoprost or timolol, both containing BAK as preservative, latanoprost-treated conjunctival specimens showed a decreased stromal collagen density and a less pronounced inflammatory infiltration than those receiving timolol, with an upregulation of MMP-1 and MMP-3 in latanoprost-treated eyes (Terai et al., 2009).

4.6. Impression cytology specimens

Developed at the end of the 1970s, impression cytology is a well-known, easily repeated technique for collecting conjunctival epithelial cells in a noninvasive, rapid, and almost painless way for biological analyses of ocular surface disorders. Using conjunctival impression, the most superficial cells of the conjunctival epithelium are collected, namely epithelial cells, mucin-secreting goblet cells, and inflammatory cells infiltrating the conjunctiva (Fig. 5) (Brignon-Baudouin et al., 2004). Classical techniques of conjunctival impression cytology allow calculation of goblet cell density and the staging of squamous metaplasia, especially in dry eye syndrome and ocular surface diseases. In glaucoma, several reports showed consistent ocular surface changes as assessed by impression cytology. In 72 patients, Brandt et al. showed statistically significant degrees of metaplasia in patients treated over the long-term and associated with the number of medications (Brandt et al., 1991). Clinical impairment of the tear film and a rapid decrease of goblet cell density were also demonstrated after starting treatment with preserved timolol (Herreras et al., 1992). Schirmer’s test and break-up time were altered compared to the basal control already significantly in the first month of treatment (P < 0.01 and P < 0.001, respectively). Similarly, impression cytology showed a progressive decrease in goblet cell density also significant at the first month (P < 0.001).

More recently, impression cytology specimens also showed ocular surface changes in glaucoma patients. Cytology scores were significantly higher in the medication group than the untreated control group. The mean scores of the control, timolol, latanoprost, dorzolamide, timolol + latanoprost, and timolol + dorzolamide groups were 0.20, 1.62, 2.00, 1.75, 2.13, and 2.44, respectively, using the Nelson’s scoring system ranging from 0 to 3 (Nelson et al., 1983). Among the medication groups, cytology scores were significantly lower in the monotherapy groups than the fixed-combination therapy groups (Hong et al., 2006).

In addition, immunocytological techniques have been developed to better assess inflammatory changes in ocular surface diseases. Our group was able to quantify HLA-DR expression in impression cytology specimens. HLA-DR antigens are normally not expressed by epithelial cells, unless in inflammatory, allergic or immune ocular surface diseases and for quantifying the level of inflammation, showing special usefulness in disorders that are clinically not or very weakly inflammatory, such as dry eye and glaucoma. In the first immunocytological study performed in glaucoma, conjunctival inflammatory antigens were investigated in impression cytology specimens from patients with or without glaucoma treatment, including 107 eyes from 55 patients with primary open-angle glaucoma. None of the untreated glaucoma eyes or controls showed reactivity for either monoclonal antibody, HLA-DR or CD23, a low affinity receptor for IgE. In contrast,
HLA-DR expression by conjunctival cells was found in 43 of 88 treated eyes (mean percentage of reactive cells, 70% ± 28%) and positive staining for the receptor to IgE in 26 of 68 eyes (52% ± 28% of conjunctival cells). The results were not related to a specific treatment or combination of antiglaucoma drugs. However, the proportion of positive specimens (3/14 for both antigens) in the group receiving chlorhexidine-containing eye drops was significantly lower than that found in the patients who had been in repetitive contact with antiglaucomatous treatments and their common preservative, BAK (Baudouin et al., 1994).

Our group further developed the technique of flow cytometry in impression cytology and demonstrated it to be a reliable method for assessing inflammatory changes in ocular surface cells. It is minimally invasive and can be routinely performed for investigating ocular surface disorders and the effects of topical drugs on the eye surface, since the technique of cell collection is the same as that used in standard impression cytology. We showed that glaucoma patients, even though clinically asymptomatic, exhibit significant overexpression of HLA-DR class II antigens (Fig. 6), ICAM-1, or interleukins IL-6, IL-8, and IL-10 in the epithelium (Baudouin et al., 2004, 2005, 2008; Pisella et al., 2004). Preserved drugs and multiple treatments reliably showed higher levels of inflammatory markers or cytokines. Interestingly, in an ex vivo and in vitro study investigating latanoprost eye drops, the only prostaglandin available at that time, we found that pro-inflammatory and pro-apoptotic effects were lower than expected, regarding the concentration of benzalkonium contained in the commercial preparation, and these effects were at even lower levels than those found in a preserved beta-blocker group (Pisella et al., 2004). We also found a relatively well-preserved goblet cell density, lower than with unpreserved beta-blocker but significantly less impaired than in the preserved beta-blocker-treated eyes. Another group similarly investigated HLA-DR in glaucomatous patients and the expression of trefoil factor family (TFF)1, MUC5AC, and HLA-DR, which were significantly higher in patients than in controls (Souchier et al., 2006). However, most interestingly, a higher MUC5AC expression and a lower HLA-DR expression were observed in patients with further successful glaucoma surgeries than in failures. Surface marker expression could therefore become a predictive factor of successful glaucoma surgery.

In a more recent study also conducted in impression cytology using a similar technique, we further investigated markers associated with the T helper (Th) 1/Th2 profiles of inflammation in various ocular surface diseases, using the expression of CCR5 and CCR4, respectively, two markers already demonstrated to reflect the two T helper pathways (Baudouin et al., 2005). In this study, vernal keratoconjunctivitis (VKC) was characterized by a high CCR4/low CCR5 profile, whereas keratoconjunctivitis sicca (KCS) had a low CCR4/high CCR5 profile, consistent with what was expected to be related to Th2 and Th1 involvements, respectively. Interestingly, we also studied a group of glaucoma patients treated over the long term and found that their conjunctiva expressed high levels of both CCR4 and CCR5, suggesting the simultaneous involvement of Th1 and Th2 pathways under the stimulation of topical drugs. To our knowledge, no other research had focused on the Th1/Th2 profile in glaucoma patients, and little information was known on the conjunctival effects of the currently available prostaglandin analogs. We therefore undertook a complementary study to improve our knowledge on the inflammatory pathways involved in glaucoma patients treated over the long term (i.e., Th1, Th2, or both) and to look for possible differences in the inflammatory profiles of the three commercially available prostaglandin analogs, namely latanoprost, travoprost, and bimatoprost. Our results first confirmed that the conjunctiva of glaucomatous patients treated over the long term was highly inflammatory, especially in patients receiving more than one eye drop (Baudouin et al., 2008). Moreover, glaucoma treatments were confirmed to act not only on CCR4, therefore most likely associated with the Th2 pathway suggesting allergic reactions, but also CCR5, the marker associated with Th1. This was found at a high level in patients receiving multiple treatments. Consequently, the Th1 and Th2 pathways seemed to be simultaneously implicated in this iatrogenic ocular pathology, resembling what has been found with other techniques in complex ocular surface diseases such as atopic keratoconjunctivitis (Metz et al., 1997). This illustrates the complexity of the mechanisms occurring in the ocular surface in glaucoma, possibly involving allergic or toxic reactions, direct stimulation of inflammatory cells, impairment of the lacrimal film, or destruction of epithelial cells and their further stimulation. Although not significant, a tendency toward slight differences between the three prostaglandins was found with HLA-DR expression, parallel to the concentrations of BAK contained in the eye drops, namely the higher the concentration, the higher the HLA-DR expression. No apparent relationship could be established with the known frequency of hyperemia, which therefore seems to correspond to a different mechanism, likely not caused by inflammatory processes as found in conjunctival biopsies (Leal et al., 2004). Interestingly, the inflammation induced by prostaglandin analogs, as assessed by HLA-DR expression in impression cytology, was also found by another group, with no significant differences between the three compounds currently available on the market, but very early after starting the treatment because increased expressions were found at 1 month (Rodrigues Mde et al., 2009).

5. Clinical evidence of preservative involvement

5.1. Prospective studies

Only very few prospective studies have addressed the question of the deleterious role of the preservative, in part because of the current lack of preservative-free compounds and to a large extent because a normal ocular surface will experience weak toxic effects after a short duration of treatment, especially with a monotherapy, that is at a low BAK exposure rate. Nevertheless, in healthy volunteers Ishibashi et al. demonstrated that preserved timolol caused significantly higher tear film instability and disruption of corneal barrier function than preservative-free timolol (Ishibashi et al., 2003). Our group found very similar results also in healthy volunteers when comparing preservative-free and BAK-containing

![Fig. 6. Comparative expression of HLA DR class II antigens using flow cytometry in impression cytology specimens in normal subjects, and glaucomatous patients treated with an unpreserved or preserved beta-blocker, or receiving multiple therapy (adapted from Baudouin et al., 2004).](image-url)
carteolol (Baudouin and de Lunardo, 1998). The tear break-up time (BUT) was significantly lower in volunteers receiving BAK. When considering that both studies were conducted in young subjects with a fully normal ocular surface, these results may help better understand the high rate of dry eye symptoms and signs in glaucomatous patients who accumulate a long duration of treatment, a higher number of medications, and an increased risk of impaired ocular surface. Indeed, one study was conducted in healthy volunteers prospectively evaluating various concentrations of BAK in tear substitutes given eight times a day for 7 days. Even a low concentration of BAK induced goblet cell loss and increased the cytoplasmic/nucleus ratio, two characteristics of dry eye disease (Rolando et al., 1991). A subsequent study prospectively assigned 132 subjects to one or two BAK-containing eye drops, BAK twice daily, or no treatment. All groups receiving BAK showed significantly decreased Schirmer test values compared with subjects not receiving therapy (Nuzzi et al., 1998).

Another more recent study used impression cytology and the technique of in vivo confocal microscopy (IVCM) to prospectively investigate in 27 eyes of 27 patients the effects of preserved or unpreserved levobunolol in the conjunctival epithelium. Patients were naive of previous antiglaucoma treatment. IVCM and impression cytology were performed before and after 6 months of therapy and showed significant differences from baseline in both groups and between the two groups. The IVCM analysis showed 61% and 17% of goblet cell density reduction from baseline, respectively, in groups 1 (preserved levobunolol) and 2 (unpreserved levobunolol) (p < 0.001). Similarly, using the Nelson’s score (Nelson et al., 1983), the grading of impression cytology parameters was significantly higher in group 1 than in group 2. Moreover, these conjunctival epithelial changes were observed only after 6 months of therapy suggesting that preservatives exert their toxic effects in a rather short period of time at least at a subclinical level (Ciancaglini et al., 2008).

5.2. Observational comparative surveys

Several studies have in recent years compared the ocular surface parameters in patients receiving preserved and unpreserved eyedrops. The ocular surface of 20 patients with open-angle glaucoma receiving 0.5% timolol (with 0.01% BAK) or bitherapy with preserved 0.5% timolol plus 1% dipivefrin were evaluated and compared with those of 20 healthy control participants receiving placebo. The Schirmer test and tear film break-up time values were significantly lower in the two treatment groups when compared with placebo (Yalvac et al., 1995). To evaluate the long-term effects of preservative-free and preservative-containing antiglaucoma eye drops on the ocular surface, a total of 84 patients were evaluated using the sophisticated method of IVCM with respect to their treatment. Significant differences were found between groups on topical BAK-containing hypotensive therapy, namely preserved beta-blocker, preserved prostaglandin, fixed or unfixed combinations of both drugs, and a preservative-free beta-blocker group. In particular, the density of superficial epithelial cells and the number of sub-basal nerves were reduced in the preservative-containing groups. In contrast, the density of basal epithelial cells, stromal keratocyte activation, and bead-like nerve shaping were higher in the glaucomatous preservative therapy groups than the control and preservative-free groups (Martone et al., 2009). Moreover this study pointed out a decreased corneal sensitivity, based on esthesiometry, in all preserved groups (ranging from 39.1 in the unfixed combination group to 49.4 in the fixed combination group) compared to control or unpreserved eyedrops (58.2 ± 1.7 and 55.8 ± 4.3, respectively, P < 0.05). No differences were found between preservative-containing groups. This study focused on corneal nerves and structural but also functional changes, with a decreased sensitivity, are quite consistent with the known denervation effects of BAK in other systems like the gastrointestinal myenteric plexus or the urinary tract (Rolle et al., 2003). This property of BAK could thus participate to the overall good comfort of patients receiving BAK-containing eyedrops, but if at least in part due to corneal hypoesthesia, this would raise new concerns about a falsely apparent good tolerance.

At a larger scale, in the above-mentioned epidemiological survey conducted in 2002 in 4107 glaucoma patients to assess the effects of preserved and preservative-free eye drops on ocular symptoms (Pisella et al., 2002), all were significantly more prevalent (about twice as much) in patients using preserved drops compared with those on preservative-free treatment. Likewise, the similarly conducted European survey also demonstrated that the incidence of ocular signs and symptoms was higher in patients receiving preserved eye drops (Fig. 4) (Jaenen et al., 2007).

5.3. Switching studies

Cross-sectional study observations have been confirmed by switching studies. In patients receiving preserved eyedrops and experiencing symptoms and signs evocative of poor tolerance, such as eyelid crusting, punctuate keratitis, or dry eye surface, changing to preservative-free eyedrops reliably leads to rapid improvement. In the Pisella study, conducted in private practices on patients treated for chronic open-angle glaucoma, ophthalmologists prescribed preservative-free eyedrops to 349 patients presenting with signs and symptoms of ocular impairment and previously treated by preserved eyedrops. After 4 months, a sizeable and significant decrease (p < 0.001) of all functional signs and symptoms was observed. A less marked improvement was also obtained in approximately 50 patients who had reduced the number of preserved eye drop solutions used with respect to the first visit. No change was observed in patients having continued their previous treatment (Pisella et al., 2002). Similarly, in the larger European survey the incidence of signs and symptoms decreased significantly by switching to a preservative-free formulation or by reducing the amount of preservative-containing treatment (Jaenen et al., 2007).

Bron and colleagues report similar results in another multicenter, prospective, open-label study conducted on 435 patients with open-angle glaucoma or ocular hypertension, by replacing a preserved solution by a preservative-free solution of the same family (timolol). After 3 months, changing to preservative-free eye drops led to a notable improvement in local tolerance, while maintaining a good pressure balance. On instillation, irritation, dry eye, or foreign body sensations and blurred vision or stuck eyelids were diminished. Between instillations, dry eye and foreign body sensations were reduced by half (15.4% vs. 8.0%). The percentage of conjunctival congestion dropped from 24.4% to 14.6%. Folliculopapillary and superficial punctate keratitis rates were also reduced by half (Bron et al., 2003).

The improvements described above are related to a significant decrease in the cytologic and inflammatory processes of the conjunctiva and the cornea (Fig. 7). In a single blind study, Campagna et al. analyzed the cytologic impression of 20 glaucoma patients before and after 3 months using preservative-free timolol in place of preserved timolol. Switching to a preservative-free solution significantly increased mucus cells and significantly improved conjunctival epithelial cell impairment (rose Bengal staining). The subjective symptoms (stinging, foreign body sensation) present on enrollment also diminished. These improvements were significant beginning at the second month of treatment. Intraocular pressure control was also maintained during the treatment change (Campagna et al., 1997). Likewise, in another
study of 21 patients treated with preserved timolol only 2 weeks of treatment with preservative-free timolol resulted in partial normalization of corneal permeability as measured by fluorophotometry (increase of +27%, p = 0.025). In parallel, the improvement or disappearance of symptoms was obtained in eight out of ten patients complaining of a burning or dry eye sensation and efficacy was not lost after removal of BAK (de Jong et al., 1994). Very recently, a new open-label multicenter switching study conducted in 158 patients treated with preserved latanoprost and experiencing signs and symptoms of intolerance showed a marked improvement when switching to preservative-free tafluprost (Uusitalo et al., in press). All symptoms and signs were significantly decreased, as well as tear break-up time increased, whereas IOP control remained unchanged after switching the prostaglandin analog. Such improvements are therefore susceptible to improve quality of life, which is known to be deeply impaired in glaucomatous patients by ocular surface-related side effects (Nordmann et al., 2003), and most likely compliance (Zimmerman et al., 2000).

6. In vitro demonstration of BAK toxicity

6.1. Chemical characteristics of BAK

Benzalkonium chloride (BAK) is a bactericidal cationic tensoactive compound used as preservative in various medical preparations in concentrations ranging from 0.004% to 0.025% in eye drops. BAK is a mixture of alkyl benzyl dimethyl ammonium chlorides with several analogs varying in the length of the aliphatic alkyl chain. In commercial preparations, the aliphatic alkyl chains possess lengths of 12, 14 and 16 carbon atoms (Gardner and Girard, 2000). At low concentrations, BAK in aqueous solutions is positively charged and possesses amphipathic conformation. These cations associate and form micelles when the concentration of BAK reaches the critical micellar concentration (CMC). Micelles are roughly spherical or globular aggregates characterized by hydrophobic core and a hydrophilic outer surface. The CMC of BAK depends on the relative composition of C12-, C14-, and C16-alkyl chains. As a consequence of micelle formation, the physical and biological properties of the solution may change abruptly, which was considered a possible cause of the abrupt termination of BAK activity at high concentrations. CMC has been reported to occur over a concentration of 0.02%. The effects of BAK micelles on ocular cells has not been fully addressed and seem to vary according to the system tested in vitro (Deutschle et al., 2006). However, even though toxicity may be less than expected in cell lines at high concentration, probably due to micelle formation, in vivo high concentrations on BAK cause major cell loss and tissue alterations (Pauly et al., 2008).

The biological activity of BAK is based on its interaction with proteins, lipids, and G proteins in biological membranes (Patarca et al., 2000). As a microbicidal agent, it is therefore highly toxic, in a time- and dose-dependent manner, widely and reliably demonstrated in a large variety of systems and organs, demonstrating not only cell toxicity but also genotoxicity and mutagenesis potential (Deutschle et al., 2006).

6.2. Models of study in ocular toxicology

One useful alternative to animal models relies on cell cultures. Of course one major limitation is the monocharacteristic of the cell monolayer that cannot mimic a more complex structure like a tissue. In particular, conjunctiva, the most reactive tissue of the ocular structure from an immunological point of view, with the complex association of epithelial cells, mucin-secreting goblet cells, dendritic cells, and intraepithelial lymphocytes, cannot be fully studied on the basis of merely epithelial cells. Nevertheless, despite this drawback, cell cultures have been widely used for investigating some pathophysiological aspects of human conjunctiva, especially for toxicological purposes. Particularly, cell lines permit easy and quick cell development and have the advantage over primary culture of being independent from conjunctival biopsy availability. Currently, the Wong-Kilbourne derivative of the Chang conjunctival cell line has been widely used for toxicological and functional in vitro studies on ocular surface diseases. This cell line has been immortalized from normal human conjunctival epithelial cells, but presents some characteristics distinguishing it from normal tissue and primary cultures, particularly in their response to inflammatory cytokines such as TNFα and INFγ (De Saint Jean et al., 2004). Indeed, like other cell lines, it has acquired some differences from normal conjunctiva in its phenotypic characteristics. However, the most common reserve to this cell line is the reported contamination with HeLa cells (Lavappa, 1978). Recently, Diebold et al. characterized the IOBA-NHC spontaneously immortalized cell line, which showed no other cell type contamination but some phenotypic differences compared to the normal conjunctival epithelium (Diebold et al., 2003). Our group evaluated the relevance of the IOBA-NHC cell line in toxicological research studies, determining whether these cell lines would be fully comparable and suitable for toxicological in vitro studies (Brasnu et al., 2008b). As expected, BAK showed similar toxicity profiles in the two cell lines. We thus confirmed the validity of using the Chang cell line for toxicological in vitro studies. However, this line seems to have a higher sensitivity...
against BAK despite their reported contamination with HeLa cells. In addition, in this study, we showed a dose-dependent toxicity of BAK on IOBA-NHC cells, validating this cell line for toxicological in vitro studies, particularly for the comparison of the apoptotic or oxidative effects of different ophthalmic medications. Nevertheless, as Chang and IOBA-NHC cell lines are in vitro models, the results obtained with each cell line cannot be fully extrapolated to in vivo conditions. In vivo, cells are protected with the action of the eyelids, the precorneal mucin layer and glycocalyx, and there is a permanent renewal of ocular surface epithelia. Furthermore, tissues have high regeneration and defense capacities and conjunctival epithelium has a stratified structure that enhances protection of the ocular surface against preservative toxicity.

6.3. Literature data

As a quaternary ammonium, the preservative BAK may act at different levels of the cell machinery and has been shown to interact with cell membranes and cell receptors, especially those involved in cell death. In Chang conjunctival cells and IOBA-NHC cells, Buron et al. demonstrated both similarities and differences between BAK toxicity and ultraviolet-induced stress (Buron et al., 2006). Both agents induced cytochrome c release from the mitochondria, caspase activation, and nuclear chromatin condensation, suggesting caspase-dependent apoptosis, prevented by stable expression of Bcl-2 protein and only partially prevented by baculovirus p35 caspase inhibitors, which more efficiently delayed the UV irradiation-induced than the BAK-induced nuclear chromatin condensation. Therefore BAK also induced caspase-independent cell death and autophagic features, likely to play a protective or at least adaptive role. Whereas both agents induced a redistribution of Fas in plasma membrane rafts and the Fas-ligand-independent formation of a death-inducing complex leading to caspase-8 activation, BAK specifically activated a caspase-independent pathway by inducing the mitochondrial release of apoptosis-inducing factor (AIF). BAK-treated cells contained autophagosomes/autolysosomes, a characteristic feature of autophagy, and siRNA-mediated downregulation of the beclin-1 gene, whose product is crucial for autophagy, increased BAK toxicity (Buron et al., 2006). In addition, a significant mutagenic effect of BAK has been found at environmentally relevant concentrations and even though no evidence of genotoxicity-related ocular side effects has been reported to date, this effect should be considered an additional potentially harmful effect in patients receiving BAK over the long term (Perk et al., 2007).

6.4. Comparative studies on preservatives

Very few comparative toxicological studies have been conducted, either in vitro or in vivo. As we developed efficient in vitro testing models in conjunctival cell lines using cold light cytofluorometry and flow cytometry, dealing with apoptosis, cell viability, cell cycle, and oxidative stress, we conducted a comparative assessment of various preservatives used in ophthalmic preparations (Debbsch et al., 2001b). BAK in three different hydrocarbon chain lengths, benzododecimine bromide (BOB), cetrimide (CET), phenylmercuric nitrate, thimerosal, methyl para-hydroxybenzoate, chlorobutanol, and EDTA were submitted to toxicological screening. Consistent toxicity was reliably found with all quaternary ammonium compounds, namely BAK, BOB, and CET, at a threshold of toxicity as low as 0.005%. Nonquaternary ammonium preservatives caused oxidative stress but to a significantly lesser extent and induced a lower cell death rate.

More recently, Epstein et al. also investigated various preservative compounds in immortalized human conjunctival and corneal epithelial cells. BAK, methyl paraben (MP), sodium perborate (SP), chlorobutanol (Cbl), and stabilized thimerosal (Thi), and EDTA with negative and positive controls were tested. The authors confirmed that all preservatives caused significant conjunctival and corneal cell toxicity, depending upon concentration. BAK exhibited more toxicity than Cbl, MP, and SP. Thi being more toxic in this model than quaternary ammoniums and EDTA and exerting minimal toxicity (Epstein et al., 2009).

6.5. Effects of BAK on conjunctival epithelial cells

Chang human conjunctival-derived cells have been widely used to demonstrate and study the toxic effects of BAK. Our group developed and used new cytotoxicity tests using cytotoxic fluorometric microtitration and flow cytometry. The techniques of MiFALC (Microtitation Fluorimetric Assays on Live Cells) allow tests on live cells, consistent with the recommendations of the European Committee for the Validation of Alternative Methods. We therefore used a series of innovative tests and methods for multiple evaluations in ocular toxicology by investigating objective biological criteria such as cellular viability, chromatin condensation, production of reactive oxygen species, and transmembrane mitochondrial potential. Therefore, many antiglaucoma drugs and their preservatives have been studied. All preservatives, even at very low concentrations, appeared cytotoxic for ocular cells and responsible for apoptosis and free radical production. Conversely, most active compounds did not induce any apoptotic mechanism; nor did they show interesting antioxidative and antiapoptotic properties (Brasnou et al., 2008a; De Saint Jean et al., 1999, 2000; Debbsch et al., 2001a,b, 2002; Guenoun et al., 2005a,b; Labbe et al., 2006b; Pisella et al., 2004).

From all these in vitro studies, it appears that BAK induces a significant, concentration-dependent decrease in cellular viability, whereas unpreserved preparations of timolol, carteolol, and tafluprost did not show any toxicity. Preparations containing the preservative induced chromatin condensation associated with an alteration of mitochondrial activity and a decrease of glutathione, suggesting an apoptotic phenomenon. ROS production was also found with preserved formulations at significantly higher levels than those observed with unpreserved drugs. Furthermore, we developed an in vitro proinflammatory model using the stimulation of intercellular adhesion molecule (ICAM)-1 using interferon (IFN)-γ in order to address the possible mechanisms leading to ICAM-1 or HLA-DR overexpression in glaucomatous patients treated over the long term. In contrast with proapoptotic cell death-promoting effects, eye drops preserved with BAK did not cause direct ICAM-1 or HLA-DR expression, but they did induce even higher ICAM-1 expression levels on IFN-γ-stimulated cells than did IFN-γ alone, whereas unpreserved drugs had no effect. This would demonstrate BAK is a costimulatory factor with inflammatory cytokines enhancing inflammatory cascade activation (Pauly et al., 2007a).

Indeed, the proapoptotic effects were seen at very low concentrations of BAK with a threshold of toxicity found at about 0.005%. Interestingly, a reliable observation found in our successive studies was the fact that commercially available prostaglandin analogs often induced a lower toxicity to conjunctival cells than the solution of BAK alone at the same concentration (Guenoun et al., 2005a,b; Pisella et al., 2004). A chemical combination reducing the amount of toxic free BAK could not be excluded. However, as the reduction of effects observed in vitro was mainly effective on free radical production, a positive antioxidative effect of the prostaglandin analog was thus hypothesized that could in part counteract the deleterious oxidative stress caused by the preservative (Fig. 8) (Guenoun et al., 2005a). This was confirmed both using the commercial formulations of travoprost and latanoprost and when testing nonpreserved latanoprost in a dose-dependent manner.
the conditions of a mucosa least in patients receiving only one drug. Despite a rather high amount of BAK instilled over the long term, at conditions we tested. This hypothesis is therefore in line with the native model to the classical Draize test (Doucet et al., 1998, 2006) basal side, yielding results that may not correlate with histo-

Guenoun et al., 2005a), whereas bimatoprost seemed not to exhibit antioxidative properties, at least in the experimental conditions we tested. This hypothesis is therefore in line with the results of clinical trials and postmarketing observations that also argue in favor of relatively good tolerance of such compounds despite a rather high amount of BAK instilled over the long term, at least in patients receiving only one drug.

6.6. Three-dimension models of corneal epithelium

In vitro experiments with immortalized cells do not really reflect the conditions of a mucosa in vivo, as isolated cells may be more sensitive than a tissue. On the other hand, a tissue exposed to various stresses may involve all cell components, which may change the response of complex tissues, such as the conjunctiva, which associates epithelial, inflammatory, and mucus cells. Such complex interactions may thus stimulate compensation modes whose result may be at the ocular surface levels a mixture of an increased cell death rate, tear film impairment, inflammatory stimulation and increased epithelial turnover. Therefore, the search for more appropriate models than cell monolayers has led to complex three-dimensional (3D) epithelial systems that are useful for toxicological purposes.

The reconstructed 3D model of human corneal epithelial cells (HCEs), supplied by SkinEthic Laboratories, was found to resemble the corneal epithelium of the human eye in morphology and thickness (Nguyen et al., 2003). It was proposed as a useful alternative model to the classical Draize test (Doucet et al., 1998, 2006) for the assessment of eye irritation potential of chemicals and cosmetic products. However, as the standard procedures are based on the MTI (3-{4, 5-dimethylthiazol-2-yl} 2,5-diphenyl tetrazo-

E-cadherin, and occludin. The BAK-induced overexpression of ICAM-1 on 3D-HCE suggests an inflammatory response observed in humans or animal models. Nevertheless, these results illustrate the tissue-level response to a toxic, most likely on an inflammatory mode in response to the cell-level death mode. Taken together, these results appear important to better comprehend the effects of low doses of a toxic compound on a more complex structure than single cells. This would fill the gap between the cell death-related observations in in vitro models and the inflammatory responses observed in humans or animal models.

However, conflicting results were also reported, minimizing the impact of preservative on ocular tissues. In contrast with our results and all the literature data on BAK toxicity, Koh-Hoeller and Jessen, in a study conducted by the Pfizer laboratory, using the same model of 3D corneal epithelium, did not find any loss of viability when testing preserved latanoprost or BAK after a 10- to 60-min contact duration (Koh-Hoeller and Jessen, 2009). This raises several important issues such as the choice of the model and the most appropriate duration of contact to best fit real-world and clinical conditions. Actually, a normal tissue in a young patient, submitted to low concentrations of a toxic agent once a day, will unlikely undergo major changes, unless treated for a very long period of time. Indeed, individual sensitivity of the tissue and accumulation of drugs will directly influence the outcome of the ocular surface over the long term. The question of the actual duration of BAK exposure to corneal cells, and subsequently the accuracy of the model, is important to consider. Koh-Hoeller and Jessen stated that BAK would only be in contact with the eye structures for few
seconds after instillation. This assumption seemed in accordance with a previous study investigating the concentration of BAK in the tears after instillation and showing rapid dilution (Friedlaender et al., 2006). However, very few data exist on ocular pharmacokinetics of BAK after instillation. Champeau and Edelhauser evaluated the presence of BAK in tissues, thus evaluating not dilution but impregnation of ocular surface tissues by the chemical compound. They found measurable concentrations in the conjunctiva and cornea up to 7 days after a single instillation in a normal rabbit eye (Champeau and Edelhauser, 1986). As BAK is usually considered a potent and useful enhancer of drug penetration into deep ocular structures, all experimental models should be considered in light of contact time, depending not on tear concentrations of BAK but instead on much more prolonged contact times, as most likely occurs with a compound that disrupts cell membranes, increases corneal epithelium permeability, and is repeatedly used over extremely long periods of time.

7. Experimental data in animal models

Chemicals, cosmetics, and pharmaceuticals have to be assessed for their irritancy potential and risk to the human eye. However, the only method accepted worldwide by regulatory authorities for the assessment of acute eye irritation potential is the Draize rabbit test (Draize et al., 1944), which has been criticized by animal welfare advocates and whose relevance, validity, and precision have been challenged because of the variability and low predictiveness of the human response (Curren and Harbell, 2002; Wilhelmus, 2001). This test is mainly based on scoring observed macroscopic changes in the rabbit cornea, conjunctiva, and iris, and various scoring systems are currently accepted (Maximum Average Score MAS, Modified Maximum Average Score MMAS, Globally Harmonized System, etc.). The rabbit model showed weaknesses for assessing nonsevere irritants and low toxics. There has therefore been an attempt to improve the sensitivity and relevance of rabbit eye models in toxicology. One major technique, in addition to standard clinical assessment and postmortem histology, is the development of corneal confocal microscopy (IVCM), an in vivo nontraumatic technique for investigating the ocular surface, first developed in humans but suitable for animal models.

7.1. The rabbit as an experimental model: recent improvements

The first toxicological study was conducted by Ichijima et al., who developed an acute stress mode suitable for mimicking the effects of long-term use of low toxicity compounds over a short period of time. Furthermore, the authors used IVCM to improve the determination of ocular surface changes following drug administration. The effects of BAK on the living rabbit cornea were studied by in vivo Tandem scanning confocal microscopy (TSCM) and

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**Fig. 9.** Immunolocalization of occludin (cell junctions), ICAM-1 (Intercellular adhesion molecule), TUNEL (late apoptosis), activated caspase-3 (early apoptosis), and Ki67 (cell proliferation) in a 3D model of corneal epithelium (Skinetics®), in control (left) or after 24 hours of contact with BAK 0.01% (right). Immunostainings appear in green. The nuclei are stained in blue with DAPI (rows 3 and 4) or in red with propidium iodide (rows 1,2 and 5). BAK was administered on the top of the epithelium for 24 hours. Results clearly show a disruption of tight junctions in the most superficial layers, namely the most exposed to BAK, overexpression of ICAM-1, late apoptosis of the most superficial layer, early apoptosis in deeper layers and a compensatory multiplication of epithelial cells corresponding to an increased turnover of the corneal epithelium (Scale bar: 150 μm).
confirmed by conventional scanning electron microscopy (SEM). Two drops of saline or phosphate-buffered saline (PBS) containing BAK in concentrations of 0.02, 0.01, or 0.005% were applied to rabbit eyes 15 times at 5-min intervals. Immediately after application of 0.02 and 0.01% BAK, no normal corneal superficial epithelial cells could be imaged by in vivo TSCM. Application of as little as 0.005% BAK caused the superficial epithelial cells to swell and desquamate. The observed desquamation of corneal superficial epithelial cells increased with higher BAK concentrations applied to the eye. One hour after final drug application, inflammatory cells appeared on the surface of the cornea treated with 0.02% BAK. These findings were correlated with SEM observations, whereas nonpreserved control solutions showed no toxicity (Ichijima et al., 1992).

Our group also used the rabbit eye for toxicological purposes, developing the most recent technical improvement of IVC M to enhance ocular surface investigations at a histological-like level of resolution. The recently developed Rostock Corneal Module of the Heidelberg Retina Tomograph (HRT/RCM, Heidelberg Engineering, Heidelberg, Germany) now allows resolutions close to 1 μm and facilitates examination of the whole ocular surface. Major applications have been developed in human diseases (Labbe et al., 2005, 2006a, 2008) and the device has been adapted to animal use (Labbe et al., 2006b; Liang et al., 2008a,b). In addition to toxicity-related changes such as corneal epithelium alterations, IVC M was able to show inflammatory infiltrates in the peripheral cornea, the limbus, and the conjunctiva, features previously only accessible to postmortem histology (Fig. 10). Furthermore, for the first time we were able to identify in vivo the conjunctiva-associated lymphoid tissue (CALT) and its major inflammatory infiltration after an acute challenge with BAK or BAK-containing eyedrops (Liang et al., 2009). Until now, no study has been carried out in vivo or even using ex vivo techniques focusing on the CALT reactions after instillations of toxic eye drops. In our toxicological model, the inflammatory cell trafficking in CALT seemed to be primarily related to the concentration of BAK because major infiltration could be observed, in a dose-dependent manner with regard to BAK concentration, and nonpreserved control eyedrops or active compounds did not cause any change. These immunoinflammatory changes in CALT, as well as in a more diffuse way in the whole conjunctiva, may participate in the strong inflammatory and apoptotic reactions observed after applications of BAK-containing eye drops, thus demonstrating the two pathways induced by BAK, namely cell death and inflammation. This approach in ocular toxicology therefore allows better investigations of low-toxicity compounds and refined assessment of active compounds or possible preservative alternatives to BAK use in this acute stress model.

Other tools for a better analysis of toxicity in rabbit eyes may also include impression cytology specimens (Liang et al., 2008a,b), another technique that allows repeated examinations before animal sacrifice, thus reducing the number of animals used for experimental purposes, in accordance with the current requirements for animal use (the Three R rule).

Nevertheless, histology, immunohistology, and electron microscopy have also widely been used for toxicological purposes and several reports have clearly confirmed the quite consistent toxicity of BAK, for any model, duration of application, and possible association with an active drug. For example, Noecker et al. found less damage and lower inflammatory scores after a 30-day application of bimatoprost, the prostaglandin analog with the lowest BAK concentration, containing 0.005% BAK, compared to timolol or latanoprost eyedrops (0.01% and 0.02%, respectively), thus confirming the reliable dose-dependent effects of BAK (Noecker et al., 2004). Consistent with this report, Kahook and Noecker counted goblet cell density after 30 days of treatment in rabbit eyes and found significantly lower density in the BAK-containing latanoprost group than in the preservative-free artificial tear group (2.21 ± 0.40 vs. 7.03 ± 1.33; p = 0.0001) (Kahook and Noecker, 2008). After a treatment of only one drop a day in healthy animals and a rather short duration, these findings must be understood in light of dramatic tear film changes and dry eye prevalence in glaucoma patients treated over the long term. They are quite consistent with human findings of reduced goblet cell density in treated patients (Herreras et al., 1992; Pisella et al., 2004).

7.2. BAK toxicity in rat

The rat is a useful model that is more suitable for handling and housing than the rabbit. It has already been used to demonstrate BAK toxicity, confirming that even in healthy animals a rather short duration of contact may exert significant histological changes that are observable in BAK-containing solutions but not with timolol alone (Baudouin et al., 1999). However, a high variability of ocular behavior in rat models may render this animal poorly reliable and less often used than the rabbit for toxicological purposes. IVC M has therefore improved the usefulness of rats and the accuracy of ocular examinations in addition to biomicroscopy and postmortem histological analyses (Labbe et al., 2006b). We performed a series of toxicological assessments of BAK in the rat eye after repeated instillations and developed IVC M techniques in rats in order to improve in vivo investigations. The effects of BAK were first evaluated using the Draize-derived criteria and irritation was only evidenced after instillation of highly concentrated BAK solutions, i.e., 0.5% and 0.25%. Then we characterized the effects of BAK using the HRT-II confocal microscope and the results showed a dose-response relationship for BAK-induced scores in rat cornea. The highest 0.25% and 0.5% concentrations caused epithelial denudation as well as major damage in the deep structures such as stromal inflammation and neovascularization as well as loss of endothelial visibility and fibrosis. The HRT-derived scores calculated from our data were therefore very high. We evidenced incomplete corneal recovery even long after toxic removal, as assessed by a decreased but still high HRT score due to anisocytosis, abnormal epithelial reflectivity and shape patterns, and persistent stromal neovascularization. In contrast with clinical data, the lower 0.01% and 0.1% BAK concentrations were characterized by a significantly abnormal total HRT score, resulting from damage mainly restricted to the epithelium that extended from cell border loss (0.01%) to epithelial erosion exposing the underlying, smaller wing cells (0.1%). The lowest 0.01% BAK concentration was a good example of a slight damage inducer, with changes restricted to the most superficial cell layers of the cornea and only identified because the most sensitive evaluation criteria were used (Pauly et al., 2007b). This detection of slight corneal changes must be seriously considered, given that it was obtained for a much shorter treatment duration than used in common treatments in humans. We therefore developed a scoring system based on IVC M findings in order to standardize toxicological changes and allow various laboratories worldwide to better evaluate low toxicity compounds using a single technique (Pauly et al., 2008).

8. Other potential issues with BAK use

8.1. Dry eye and ocular surface diseases

BAK is a well-known irritant in dermatological and allergological investigations, but it is rarely recognized as the main allergen responsible for contact dermatitis (Uter et al., 2008). However, by truly allergic mechanisms or as a cofactor and an irritant compound it may cause or enhance various clinical manifestations at the ocular surface level, such as allergic or nonallergic blepharitis, meibomian
In vivo confocal microscopy (IVCM) images of rabbit cornea superficial epithelium (column 1) and CALT structure (column 2), 4 hrs after 15 instillations of various antiglaucoma prostaglandin analogs. After instillations of PBS (A), BAK-free Tafluprost (B), or BAK-free travoprost (Travatan Z, C), rabbits presented a normal corneal epithelium with a regular polygonal mosaic appearance and brightly reflective nuclei. They did not induce any obvious changes in CALT structure. However, the three BAK-containing solutions, 0.02% BAK + latanoprost (D), 0.015% BAK + travoprost (E), and to a lesser extent 0.005% BAK + bimatoprost (F) induced partial desquamation of epithelial cells, with some epithelial cells showing an irregular aspect with hyperreflectivity patterns or a loss of cell borders (column 1). They also induced substantial gland dysfunction, chronic conjunctival inflammation, or tear film instability. Through its toxic and proinflammatory effects, BAK may therefore cause or aggravate dry eye disease and allergic conjunctivitis. The tear film is a fundamental protective layer for the ocular surface through its lubricating, mechanical, trophic, and antimicrobial properties. Goblet cells produce soluble mucins and participate in tear film stability. These cells are extremely sensitive to toxic and inflammatory stresses. They have been shown in humans to be decreased in density after short exposure to BAK (Rolando et al., 1991) or BAK-containing timolol (Herrerias et al., 1992). Long-term exposure to preserved antiglaucoma drugs also caused decreased goblet cell density (Pisella et al., 2004). In animal models, Wilson and colleagues demonstrated that 0.01% BAK caused precorneal tear film disruption in rabbits (Wilson et al., 1975). More recently, an experimental study conducted in albino rabbits showed significantly greater reduction in the tear film break-up time in animals receiving a preserved beta-blocker compared with those given a nonpreserved beta-blocker (Pisella et al., 2000). At the biological level, MUC1 and MUC16 were found to be reduced after exposure to BAK in human corneal-limbal epithelial cells and human corneal epithelium. Transmission electron microscopy of the anterior corneal surface revealed fixation of the mucus layer after exposure to 0.01% BAK for 5 or 15 min, whereas more prolonged exposure (60 min) to 0.01% BAK destroyed the mucus layer (Chung et al., 2006).

In addition, as a tensioactive compound, BAK is also a detergent for the lipid layer of the tear film, causing evaporation of the aqueous tear film. The result of both mechanisms, namely mucus and lipid-layer alterations, is a globally impaired tear film with tear instability and excessive evaporation, hallmarks of dry eye disease (Dry Eye WorkShop, 2007a). For example, in humans, a decreased break-up time was found after a short duration of treatment with BAK-containing carteolol in healthy volunteers (Baudouin and de Lunardo, 1998) and increased corneal epithelial permeability of dry eye patients was shown in dry eye with additional impairment when using artificial tears containing BAK compared to non-preserved eyedrops (Gobbels and Spitznas, 1989, 1992).

Through the loss of its protective properties, an impaired tear film will cause dry eye symptoms and corneal damage and also convey cytotoxic inflammatory mediators throughout the ocular surface. Tear film alterations may even stimulate a series of biological changes in the ocular surface leading to subsequent neurogenic inflammation and further impairment of the tear film, actually creating a vicious circle (Baudouin, 2007; Dry Eye WorkShop, 2007a).

8.2. The crystalline lens: is BAK an inducer of cataract?

The use of antiglaucoma medications has also been associated with the development of cataract. In the Ocular Hypertension Treatment Study (OHTS) an increased rate of cataract extraction and cataract/filtration surgery was observed in patients receiving medication (7.6%) compared with the non-treated control group (5.6%), with an odds ratio of 1.56 (Herman et al., 2006). The Blue Mountains Eye Study also showed that antiglaucoma therapy seems to increase the risk of cataract formation (odds ratio of 1.90) (Chandrasekaran et al., 2006). These observations raise the hypothesis that antiglaucoma treatment may increase the lens inflammatory cell infiltration in the CALT structure, in the follicle area but at an even higher level in the parafollicle areas, consisting of lymphocyte-like and dendritiform cells. Quantification of epithelial changes and cell infiltrates clearly showed a dose-dependent effect of BAK (0.02% BAK + latanoprost > 0.015% BAK + travoprost > 0.005% BAK + bimatoprost). All images: 400μm X 400μm; numbers on the figures show depth from the surface.
interleukin-1 in expression by lens epithelial cells of soluble mediators involved in a much lesser extent latanoprost or timolol, strongly stimulated the epithelial cell line. The results showed that BAK alone, and to a much lesser extent latanoprost or timolol, strongly stimulated the expression by lens epithelial cells of soluble mediators involved in inflammatory and/or apoptotic processes, i.e., prostaglandin E2, interleukin-1α, and interleukin-6 (Goto et al., 2003). These mediators could therefore favor or stimulate lens opacification.

8.3. Trabecular meshwork: raising new hypotheses

The glaucomatosus trabecular meshwork is characterized by a dramatic trabecular cell loss, increased trabecular extracellular matrix, and accelerated senescence, resulting in increased outflow resistance. The mechanisms of trabecular cell loss in glaucoma patients are still poorly understood. A possible role for oxidative stress has been proposed and since BAK could stimulate oxidative stress, cell death, and fibronectin mRNA in an in vitro model of trabecular cells, the involvement of BAK in trabecular meshwork degeneration was therefore hypothesized (Yu et al., 2008). Likewise, Samples and colleagues demonstrated that BAK caused a significant inhibition of the growth of trabecular meshwork cells at extremely low concentrations (Samples et al., 1989). To determine whether drug-induced apoptosis could be one of the mechanisms by which trabecular cells die in glaucoma, we evaluated the effects of BAK-containing or BAK-free antiglaucoma medications on cell cytoskeleton and apoptotic marker expression in two cultured human trabecular meshwork cell lines (Fig. 11). In a 1/100 dilution, i.e., an extremely low concentration, unpreserved beta-blockers had no apoptotic effect, preserved beta-blockers and latanoprost significantly increased expression of only Apo2.7, an early apoptosis marker, while BAK significantly increased all the apoptotic markers investigated. When tested in a 1/10 dilution, all drugs except unpreserved timolol triggered a 2- to 3.5-fold increase in apoptotic patterns, whereas up to 95% of the cells underwent apoptosis upon treatment with BAK, representing a nine fold increase over the background level (Hamard et al., 2003). The most toxic concentrations seemed slightly higher than those assumed to be found in the aqueous humor after instillation (supposedly corresponding to a 1/100 dilution). However, repeated instillation may cause further involvement of trabecular cells submitted to long-term treatments and the impact of toxics on diseased trabecular tissues have not been evaluated. Moreover, BAK was shown to accumulate in conjunctival space since only a single drop of BAK could cause measurable amounts in the conjunctiva up to 7 days after instillation (Champeau and Edelhauser, 1986). No data are available for the trabecular meshwork, but it is most likely that BAK accumulates in deep tissues after prolonged administration and the aqueous humor route is probably not the only one to cause contact of BAK with trabecular tissues. In addition, the impact of inflammatory and toxic reactions, resulting from chronic treatments with BAK-containing eyedrops over the long term, remains to elucidate. From all the above-mentioned studies and surveys, in humans, in experimental models, and in vitro, it can be assessed whether conjunctival epithelial cells are involved as targets and/or stimulators, in major inflammatory changes such as overexpression and/or synthesis of class II antigens, adhesion molecules, chemokines, chemokine receptors, interleukins, or cell death markers and mediators. Additionally, the substantia propria of the conjunctiva is infiltrated by inflammatory cells (Baudouin et al., 1999; Broadway et al., 1994a) and at least in the rabbit eye where it can be assessed in vivo, CALT follicles are stimulated by repeated instillations of BAK-containing eyedrops. Therefore, the impact of high rates of cell death and inflammatory signals on deeper but still very close structures such as the trabecular meshwork cannot be excluded and this hypothesis deserves further attention.

This would indeed raise the new and ominous hypothesis that BAK present in antiglaucomatous treatments could stimulate trabecular senescence, resulting in further damage to the trabecula with a possible impact on outflow and consequently IOP, at the same time as drugs are precisely aimed at decreasing IOP.

8.4. Retinal involvement

Several antiglaucoma eye drops have been reported to cause cystoid macular edema following cataract surgery. Although prostaglandin analogs used as antiglaucoma treatment would be more prone to cause this complication, the association with other compounds such as timolol raised the hypothesis of a role played by the common preservative BAK. Therefore, after a series of investigations on the possible causes of macular edema following cataract extraction and the role of antiglaucoma drugs and their preservative, a clinical trial was conducted by Miyake et al., comparing the impact of preserved timolol with preservative-free timolol. The authors found significantly greater aqueous flare and a higher opacity rate and/or accelerate cataract formation (Brandt, 2003). No evidence of a specific role for one or another compound could be found, which again argues for a possible interaction with the common toxic compound found in eye drops, namely the preservative that accumulates in the eyes of patients treated with several drugs over the long term. Indeed, Goto and colleagues investigated the effects of latanoprost, timolol maleate, and BAK in a human lens epithelial cell line. The results showed that BAK alone, and to a much lesser extent latanoprost or timolol, strongly stimulated the expression by lens epithelial cells of soluble mediators involved in inflammatory and/or apoptotic processes, i.e., prostaglandin E2, interleukin-1α, and interleukin-6 (Goto et al., 2003). These mediators could therefore favor or stimulate lens opacification.

Fig. 11. Cultured trabecular cell line immunostained with alexa-phalloidin (green staining of actin) and propidium iodide (red staining of nuclei). Degeneration of trabecular cells following 15 min of exposure with 0.01% BAK; A. Normal control cells; B. Total disorganization of cell cytoskeleton; C. No effect of unpreserved timolol. The scale bars indicate 25 μm (Adapted from Hamard et al., 2003).
incidence of angiographic cystoid macular edema after cataract surgery in the preservative-containing group. Based on the findings of these studies, which indicate that the preservative causes increased synthesis of proinflammatory mediators and intensified postoperative inflammation, the term pseudophakic preservative maculopathy was proposed (Miyake et al., 2003).

8.5. Nonocular side effects

As the volume of the preocular tear film is much lower than the volume of one instilled drop, the excess volume may reach the nasopharyngeal mucosa, resulting in a significant systemic absorption, specially when the patient instills more than one drop, which is quite common, either due to missing eye drops or simply because about 50% of patients are treated with two or more antiglaucomatous drugs, in both eyes. In such cases, the effects of systemic absorption or even of BAK in the bronchial tract are certainly underestimated. In the late 1980s, a series of studies demonstrated that BAK was a potent bronchoconstrictor agonist when inhaled by patients with asthma in concentrations similar to those in which it was present in commonly used bronchodilator solutions (Beasley et al., 2001). BAK was only eight times less potent as a bronchoconstrictor agonist than histamine and it was shown to cause bronchoconstriction through a combination of mast cell activation and stimulation of neural pathways. It has been shown that the inclusion of BAK not only reduces the overall bronchodilator efficacy of the product, but also causes marked bronchoconstriction in a significant proportion of patients with asthma, whereas preservative-free bronchodilators did not show these paradoxical effects. Therefore Beasley and colleagues stated: “When considered together with previous studies, these findings indicate the urgent requirement for the worldwide withdrawal of BAK from bronchodilator nebulizer solutions” (Beasley et al., 2001). The impact of BAK contained in eye drops on respiratory mucosae must still be evaluated, but in light of pneumological assessments on BAK, it could be hypothesized that repeated instillations of BAK, alone or in interaction with the active compound, e.g., beta-blockers or prostaglandin analogs, could play a role in the respiratory side effects experienced by some glaucomatous patients.

9. Are there alternatives to BAK?

9.1. Eliminating the preservative

Considerable efforts have been made in the recent past by the pharmaceutical industry to develop new antiglaucomatous compounds that would bring about efficacy, safety, and compliance. As BAK toxicity is mainly dose-dependent, reducing the number of instillations has improved ocular tolerance. Long-acting preparations of timolol and prostaglandins are given once a day, mathematically reducing the amount of BAK in contact with the eye by 50%. The same has occurred for various fixed combinations of timolol and prostaglandins, carbonic anhydrase inhibitors, and brimonidine, which are commercially available in many countries. Little information is available on the safety and tolerance of fixed combinations compared to their unfixed counterparts. As expected, it appears that the fixed combinations have a better safety profile than the unfixed association (Hommer, 2007), but all the studies were conducted in selected patients for a short duration and may not reflect the actual effects of reducing preservatives. We conducted a preliminary in vitro toxicological study aimed at comparing associations of eye drops, which raises technical issues and explains the scarcity of such evaluations, confirming the lower proapoptotic and oxidative effects of fixed combinations of timolol/brimonidine and timolol/bimatoprost compared to their unfixed counterparts (Mehana, submitted for publication).

Nevertheless, the only way to totally eliminate BAK-related side effects, especially in the most sensitive patients, would obviously be to remove BAK from eye drops; however, this raises industrial and regulatory concerns. Single-dose units are the most frequently used preservative-free preparations and depending on the country, some compounds are available, mainly beta-blockers, mostly because they came first to generics and could easily be developed. However, several drawbacks have been pointed out regarding single-dose units, such as higher cost and difficult handling, especially in elderly patients. Multidose bottles have therefore been developed, either by allowing preservative filtration through and adsorption on a porous membrane or by using a valve system that hinders penetration of bacteria into the bottle. The ABAK® (Laboratoires Théa, France) and COMOD® (Ursapharm, Germany) systems have thus been patented and commercialized with various beta-blockers such as timolol, carteolol, and nonantiglaucomatous compounds (Meloni et al., 2009; Pisella et al., 2000, 2004, 2000). The same absence of toxic effects was confirmed both in vitro and in an experimental acute challenge in rabbit eyes with preservative-free tafluprost, which showed no significant clinical, histopathological, apoptotic, or inflammatory effect (Brasnou et al., 2008a; Liang et al., 2008a).

More recently, an unpreserved fixed combination of timolol and dorzolamide (Merck, Sharpe & Dohme, NY) and a prostaglandin analog, tafluprost (Santen, Japan), have been developed and approved in several countries.

9.2. Is the active compound neutral or does it interact with the preservative?

Given that very few compounds have been developed in preservative-free formulations, most of the information available on the possible interactions with the active compounds is available based on in vitro or experimental studies. Almost all currently available data have clearly confirmed that beta-blockers have no toxic effects on their own (Baudouin et al., 1999; De Saint Jean et al., 2000; Pisella et al., 2000, 2004). The same absence of toxic effects was confirmed both in vitro and in an experimental acute challenge in rabbit eyes with preservative-free tafluprost, which showed no significant clinical, histopathological, apoptotic, or inflammatory effect (Brasnou et al., 2008a; Liang et al., 2008a).

However, in addition to the simple neutrality of preservative-free compounds, a possible specific effect of prostaglandin analogs has been proposed in several in vitro assays. Indeed, in a conjunctival cell line we found that two preservative-containing prostaglandins, latanoprost and travoprost, appeared slightly but consistently less toxic than their BAK counterpart, through a protective mechanism perhaps related to the antioxidant properties of prostaglandins (Guennoun et al., 2005a). These results were confirmed by Yu et al. in cultured human trabecular cells by showing a reduced effect of BAK in the presence of prostaglandin analogs, through antioxidative effects that would counteract the pro-oxidative and toxic properties of the preservative (Yu et al., 2008).

Another specific positive effect of the prostaglandin agonist could target goblet cells that are known in other systems to be stimulated by prostaglandin derivatives. In the human eye, interesting studies using impression cytology have shown a relatively lower decrease in goblet cell density compared to preserved timolol, despite a higher concentration of BAK in the prostaglandin solution (Pisella et al., 2004), or a transient increase in goblet cell density after a short duration of treatment before goblet cell density decreased after longer periods of treatment, likely as
a consequence of the delayed cytotoxic effects of the preservative over the long-term (Moreno et al., 2003).

Therefore, when administering a drug that contains both a toxic and an antitoxic compound, the final result may be very confusing, especially since the prostaglandin in all experiments was not able to totally counteract the detrimental effects of BAK. This would further argue in favor of definitively eliminating the toxic compound in order to better evaluate the other properties of the active drug besides their IOP-reducing effect.

9.3. Vehicle/preservative interactions

To reduce the impact of the preservative, several attempts have been made to provide vehicles that would interact with BAK to limit its toxic effects. Although no data are clinically available, in vitro studies showed that carbomer and hyaluronates, two widely used compounds in tear substitutes, can decrease BAK toxicity compared to the solution. In Chang conjunctival cells, Debbasch et al. investigated the cytotoxicity of low concentrations of unpreserved or preserved carbomer 934P (0.03% and 0.3%), unpreserved or preserved hyaluronic acid (0.018% and 0.18%), and BAK (0.0005% and 0.005%) for 15 min or for 15 min with 24 h of cell recovery, using cold light fluorimetry and confocal microscopy (Debbasch et al., 2002). The authors found that cytotoxicity of the two tear substitutes was significantly lower than that observed with BAK alone, although the same concentrations of preservative were used. Moreover, these two ophthalmic hydrogels seemed to possess antioxidative properties. Consistent with these results, in a more recent study, high-molecular-weight hyaluronan was able to decrease the toxic effects of BAK in epithelial cell lines, reducing both apoptosis and the oxidative effects of BAK, through an interaction between BAK and the vehicle or by blocking cell death receptors at the cell membrane level (Pauloin et al., 2008).

Another approach to reducing BAK cytotoxicity is to combine the quaternary ammoniums that are highly lipophilic and positively charged in an emulsion of lipids. Recent in vivo studies conducted in rabbits in our laboratory have demonstrated the role played by cationic emulsions in reducing ocular cytotoxicity induced by quaternary ammonium-containing solutions (Liang et al., 2008b). Repeated instillations of a cationic emulsion formulation containing 0.02% BAK on the ocular surface of the rabbit induced fewer changes of the ocular surface microstructures and a lower expression pattern of inflammatory markers than did 0.02% BAK in solution. Likewise, the same model also demonstrated that cationic emulsions with 0.002% cetalkonium, another quaternary ammonium, were not toxic for the ocular surface and displayed findings quite similar to those found in the control group (Fig. 12) (Liang et al., 2008b). Moreover, compared with traditional

Fig. 12. Immunostaining of inflammatory (CD45, green staining, lines 1 and 2) and apoptotic (TUNEL, green staining, lines 3 and 4) markers in rabbit eye cryosections after repeated instillations of an ophthalmic solution (15 instillations in 90 minutes). A. PBS; B. BAK Solution (Sol); C. BAK Cationic Emulsion (Em); D. Cetalkonium solution (CKC Sol); and E. CKC Emulsion, at Day 1. Nuclei are stained in red with propidium iodide. Immunohistology clearly showed that repeated instillations of BAK solution induced numerous CD45+ inflammatory cells and TUNEL+ apoptotic cells infiltrating the limbus and conjunctival areas. Comparatively BAK Em and CKC Sol induced moderate infiltrates, with significantly less inflammatory/apoptotic cells. Only occasional inflammatory/apoptotic cells were found after CKC Em or PBS instillations. These experimental approaches clearly demonstrated that ocular surface toxicity was reduced by using an emulsion instead of a traditional solution. The scale bars indicate 100 μm (adapted from Liang et al., 2008b).
solutions, the emulsion optimized the ocular surface homeostasis by its oily properties, since the emulsion alone was developed as a tear substitute for dry eye symptoms (Cationorm®, Novagali Pharma SA, France). Since lipid compounds have been proposed as tear substitutes with good relief of patient symptoms and signs, this approach to improving the vehicle can be proposed for glaucoma patients, especially for those suffering from ocular surface diseases such as dry eye or allergy. Indeed, an emulsion of latanoprost was found not to be toxic in the acute challenge model in rabbits compared to BAK-containing prostaglandin analogs (Liang et al., 2009).

9.4. Non-/less toxic preservatives

Extensive research has been conducted to discover and develop less toxic preservatives than BAK and quaternary ammoniums. However, since a preservative must be a potent antimicrobial agent while not being cytotoxic, only very few agents have been proposed and are commercially available. Noecker and colleagues investigated in rabbit eyes the effects of Purite®, a stabilized oxychloro complex, compared with topical BAK-containing antiglaucoma medications. After 30 days, both in the cornea and the conjunctiva, BAK-containing dorzolamide, timolol, and latanoprost produced significantly more changes than did artificial tears containing Purite® and brimonidine Purite® ($p = 0.001$). In this study, treatment with glaucoma medications that contained higher levels of BAK resulted in greater damage of the cornea and conjunctiva compared with preparations preserved with lower concentrations of BAK, consistent with all previous studies conducted by our group (Noecker et al., 2004). Purite® was also shown to be effective in enhancing penetration of the active compound brimonidine into aqueous humor (Dong et al., 2004), which reduced the brimonidine concentration from 0.2% to 0.15% without significant loss of IOP-reducing effects. Clinically, the two changes — moving from BAK to Purite® and reducing brimonidine concentration — resulted in significantly better tolerance, especially in irritated eyes (Mundorf et al., 2003).

Another approach has been developed for avoiding BAK as a preservative in a prostaglandin analog solution, travoprost, which exists both preserved with BAK and with a new preservative system, Sofzia®, composed of boric acid, propylene glycol, sorbitol, and zinc chloride. In randomized clinical trials, BAK-free travoprost reduced IOP similarly to BAK-containing travoprost, with a slight reduction of hyperemia (Lewis et al., 2007), thus further demonstrating that BAK is not absolutely mandatory to obtain appropriate penetration of the active compound into the anterior chamber. We carried out a toxicological study using our previously validated protocols in conjunctival cells and confirmed that BAK-free travoprost induced significantly less apoptosis and fewer alterations of cell viability and membrane integrity than did BAK-containing latanoprost or travoprost (Baudouin et al., 2007). In animal models, a once-daily application in the rabbit eye also caused significantly less corneal epithelium damage as assessed by transmission electron microscopy and less conjunctival lymphocyte infiltration than latanoprost preserved with BAK, with no differences with the control eyes receiving artificial tears (Kahook and Noecker, 2008). The same authors also compared changes in the number of goblet cells after chronic exposure to latanoprost preserved with 0.02%...
BAK to travoprost preserved with Sofzia® or preservative-free artificial tears and found a dramatic decrease of goblet cells in the BAK-receiving group (2.21 ± 0.40 per high-power field), contrasting with the values found in the Sofzia® group, which were not only nonsignificantly different from those found in the control group (6.02 ± 1.20), but even slightly higher after only 1 month (7.03 ± 1.33, nonsignificant), which may corroborate a possible stimulatory effect of the prostaglandin analog to goblet cells (Kahook and Noecker, 2008).

In an open-label study, 691 patients treated with latanoprost or bimatoprost switching to BAK-free travoprost due to tolerability issues demonstrated significant improvement of ocular surface symptoms and hyperemia, without reducing or even improving IOP control (Henry et al., 2008).

A third candidate as a preservative that could be a less toxic alternative to BAK is polyquaternium, a family of polyacetic polymers that are used in the personal care industry. At least 37 different polymers exist under the polyquaternium designation but polymers that are used in the personal care industry. At least 37 polymers may deeply impact patients of antiglaucoma medications should not be neglected because they may become one of the key objectives of glaucoma therapy. There- fore, for patients who are highly sensitive to preservatives because of preexisting or concomitant ocular surface diseases, for patients receiving combinations of two or more drugs, for those who are at risk of undergoing surgery, and finally for all patients who will need treatments over several decades, preservative-free formulations would provide clinically relevant benefits and should become a gold standard in ocular pharmacology in the near future.

References


