Antiseptics and the Ocular Surface: In Vitro Antimicrobial Activity and Effects on Conjunctival and Corneal Epithelial Cells of a New Liposomal Ocular Spray Containing Biosecur® Citrus Extract

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ABSTRACT

Introduction: The study aimed to evaluate the in vitro antimicrobial activity of a new liposomal ocular spray containing the antiseptic Biosecur® citrus extract (Oftasector, OFF-HEALTH, Florence, Italy) and its in vitro effects on cultured human corneal and conjunctival cells.

Methods: Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of Oftasector against Candida albicans and Gram-positive and Gram-negative bacteria, including antibiotic-resistant strains, were determined. Human corneal and conjunctival epithelial cells in vitro were incubated for 10 and 30 min with Oftasector or its components. The cytotoxicity was assessed through the release of cytoplasmic enzyme lactate dehydrogenase (LDH) into the medium; the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the cell viability.

Results: Oftasector was active at dilutions ranging from 1:2 to 1:16 and it displayed bactericidal and fungicidal effect against all assayed microorganisms. Most of the reduction of Staphylococcus epidermidis vitality (65%) occurred within the first minute of exposure. The cytotoxicity of Oftasector was similar to its vehicle, and the cell viability was significantly reduced only by Oftasector in its undiluted form. Conversely, Biosecur induced a significant cytotoxicity in all the experiments.

Conclusion: Oftasector showed a rapid and wide-spectrum antibacterial activity, with an optimal in vitro tolerability profile.
Keywords: Antiseptic; Biosecur; Citrus Extract; Conjunctival Epithelial Cells; Corneal Epithelial Cells; Flavonoids; Liposome; Oftasecur

Key Summary Points

Why carry out this study?
As a result of the increase in antibiotic resistance in ocular bacterial strains caused by the excessive use of antibiotics in the treatment and prophylaxis of ocular infections, interest in antiseptics in ophthalmology is growing. Nevertheless, antiseptics can be toxic for the ocular surface, especially at high concentrations.

A new antiseptic liposomal ocular spray containing citrus bioflavonoids, Oftasecur, has been recently introduced onto the market.

The aim of this study was to evaluate the in vitro antimicrobial activity of Oftasecur on the bacterial strains that are among the most common causes of ocular infections, and its effects on cultured human corneal and conjunctival cells.

What was learned from the study?
Oftasecur showed a rapid and wide-spectrum antibacterial activity, with a promising in vitro tolerability profile.

Our data support a recent pilot clinical study on candidates for intraocular injections that found a significant reduction in conjunctival bacterial load after a 4-day prophylactic treatment with Oftasecur Ocular Spray, but further studies are necessary to confirm these results on a larger number of patients.

INTRODUCTION

Eye infections, and especially endophthalmitis, can have a devastating effect on the patient’s visual function [1]. As a result of the increase in antibiotic resistance in ocular bacterial strains caused by the excessive use of antibiotics in the treatment and prophylaxis of ocular infections [2], interest in antiseptics in ophthalmology is growing [3–6]. Nevertheless, antiseptics can be toxic for the ocular surface, causing corneal epithelial defects or epithelial damage, especially at high concentrations [7]. Therefore, research is focusing on topical formulations containing antiseptics with a wide antimicrobial spectrum, but with low toxicity for the ocular surface.

Among antiseptics, flavonoids are natural compounds that display antioxidant, free radical scavenging, and various biological activity [8, 9]. Specifically, flavonoids extracted from the peel of citrus fruits have shown a large spectrum of antibacterial activity both against Gram-negative and some Gram-positive bacteria [10, 11], paving the way for the introduction in food processing industry of Biosecur®, a non-toxic organic, alcohol-free surface cleaner containing citrus bioflavonoids dissolved in food grade glycerin [9].

Recently, a new Biosecur liposomal commercial formulation, Oftasecur Ocular Spray® (OFFHEALTH S.p.a., Florence, Italy) containing 0.2% Biosecur, 0.15%, hypromellose, 1%, phospholipids S80, boric acid, sodium tetaborate decahydrate, sodium chloride, and distilled water was introduced onto the market [12]. This ocular spray works by delivering the molecule droplets to the eyelid margin, so that the drug mixes with the tear film at the eyelid opening.

In this in vitro study, we evaluated the antimicrobial activity of Oftasecur on the bacterial strains that are among the most common causes of ocular infections, and its effects on cultured human corneal and conjunctival cells.

METHODS

This study does not contain any experiment with human participants or animals performed by any of the authors.
In Vitro Antimicrobial Activity of Oftasecur

Microbial Strains
Three reference Gram-positive bacteria, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 35984, *Streptococcus pyogenes* ATCC 19615, two reference Gram-negative microorganisms, *E. coli* ATCC 27325, *Pseudomonas aeruginosa* ATCC 15442, and one yeast, *Candida albicans* ATCC 10231 were used. Two clinical isolates were also tested: a methicillin-resistant *Staphylococcus aureus* (MRSA), clinical isolate 1 (CI-1), and a multidrug-resistant (MDR) *Staphylococcus epidermidis* clinical isolate 2 (CI-2). The clinical isolates were collected from specimens submitted to the Clinical Microbiology Laboratory of the Pisa University Hospital and used anonymously. The bacterial strains were routinely grown on Mueller–Hinton agar (MHa) (Oxoid, Thermo Fisher Scientific, UK) at 37°C, and *C. albicans* was grown on Sabouraud dextrose agar (SDA; BioMérieux, Marcy l’Étoile, France) at 30°C.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Determinations
MIC and MBC were determined in order to assess antimicrobial activity of Oftasecur against Gram-positive and Gram-negative bacteria, as well as *C. albicans*. A 1% liposome solution prepared with all components of Oftasecur except Biosecur (LP) was used as control. Oftasecur Ocular Spray and the control formulation were kindly provided by OFFHEALTH S.p.a. (Florence, Italy). An appropriate volume of Oftasecur was nebulized into a sterile tube before each experiment. The nebulized compound and the control solution were immediately used. Microdilution assays were performed according to the European Committee on Antimicrobial Susceptibility Testing standards (EUCAST 2017) in a 200-µL volume/well. To prepare 96-well plates, Oftasecur and LP were twofold serially diluted in cation-adjusted Mueller–Hinton broth (CAMHB) (BD) containing Ca²⁺ (100 mg/L) and Mg²⁺ (35 mg/L) for the bacterial antimicrobial assay and in RPMI 1640 medium with l-glutamine but without bicarbonate, buffered to pH 7.0 with 0.165 M 3-(N-morpholino)ethanesulfonic acid (Trek Diagnostic Systems, Inc., Thermo Fisher Scientific, UK) for *C. albicans* assay. Inocula were prepared from a single colony collected from MHa or SDA by diluting microbes in sterile water adjusting turbidities to a density of 0.5 McF (1–2 × 10⁸ CFU/mL for bacteria, about 10⁶ CFU/mL for *C. albicans*). Then, bacteria were diluted in CAMHB to a final concentration of 0.5–2.5 × 10⁷ CFU/mL and *C. albicans* was diluted in RPMI 1640 medium to a final concentration of 0.5–2.5 × 10³ CFU/mL. Inocula (50 µL) were combined 1:1 with the solution of products and culture medium in 96-well microdilution plates. Plates were incubated at 37°C for 24–48 h for all the strains. MIC were determined following the EUCAST reading guide (EUCAST, 2017). Plates were read visually, and MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited microbial growth detected by the unaided eye. Plate reading was aided by comparing the turbidity of test wells with positive and negative control wells. Then, 100 µL of the suspensions obtained by the MIC assay at the MIC concentration of the two products and at concentrations higher than the MIC values were seeded onto blood agar plates for bacteria, or SDA for *C. albicans*, to evaluate MBC values. Plates were incubated at 37°C for 24 h to determine the number of CFU. The MBC was defined as the lowest concentration of product killing at least 99.9% of viable microorganisms after 24 h incubation. All susceptibility tests and MBC determination assays were performed in triplicate.

Time-Kill Test
In vitro bacterial killing assays were performed against *S. epidermidis* ATCC 35,984, the bacterial species most commonly associated with ocular infections [13]. Bacteria were suspended in 10 mM sodium phosphate buffer (NaPB, pH 7.4) to approx 10⁷ cells/mL. Suspensions were diluted to 5 × 10⁵ CFU/mL in 4 mL Oftasecur. At the same time, controls were prepared in NaPB. After 1, 10, 45, and 60 min of incubation at 37°C, bacterial suspensions were diluted with...
PBS and plated onto Luria Bertani (LB) agar plates. After incubation at 37 °C for 24 h, colony-forming units (CFUs) were counted. Three independent experiments were performed in duplicate.

**In Vitro Effects of Oftasecur and Its Components on Cultured Corneal and Conjunctival Epithelial Cells**

Human corneal epithelial cells (HCE-2) [50B1] (ATCC CRL-11135) were obtained from ATCC company (American Type Culture Collection, Manassas, USA). Human conjunctival epithelial cells (HConEC) and corneal epithelium cell medium were provided by Innoprot (Derio, Bizkaia, Spain). Keratinocyte serum-free medium, bovine pituitary extract (BPE), and epidermal growth factor (EGF) were purchased from Gibco-BRL (San Giuliano Milanese, MI, Italy). Hydrocortisone, insulin, fibronectin, bovine collagen type I, bovine serum albumin (BSA), penicillin/streptomycin (P/S), bovine fetal serum, poly-L-lysine, benzalkonium chloride (BAK), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were provided by Sigma (St Louis, MO, USA). The cytoxicity detection kit (lactate dehydrogenase, LDH) was purchased from Roche Diagnostics (Basel, Switzerland).

Oftasecur and all its constituents were a kind gift from OFFHEALTH S.p.a. (Florence, Italy).

Institutional review board approval was not required at our institute for the use of immortalized cell lines.

**Human Corneal Epithelial Cells (HCE-2)**

Human corneal epithelial cells (HCE-2) were cultured in keratinocyte medium that was changed twice a week and were incubated at 37 °C in an atmosphere of humidified air and 5% CO₂ at 37 °C as previously reported [6].

**Incubation with Drugs**

Human corneal and conjunctival epithelial cells were resuspended and seeded into 24-well plates. When they reached 70–80% confluence, they were exposed to Oftasecur or to the constituents of the formulation (Biosecur 0.2%; a 1% liposome solution without Biosecur, LP; borate buffer, BB) dissolved in phosphate buffered saline (PBS). The vehicle (PBS) was used as positive control and BAK 0.01% was used as negative control for maximum cell death.

Conical and conjunctival epithelial cells were incubated with drugs at different concentrations (dilution 1:1, 1:2, 1:4, and 1:32) and for two different periods (10 and 30 min).

**Analysis of Cell Viability: MTT Assay**

The viability of the conjunctival and corneal epithelial cells exposed for two time periods (10 and 30 min) to the complete formulation and different constituents of Oftasecur in different dilutions (1:1, 1:2, 1:4, and 1:32) was evaluated by MTT assay, as previously described [6].

The vehicle was used as a positive control. Cell viability was expressed as a percentage of the cells incubated in the vehicle in the corresponding exposure period.

**Evaluation of Cell Death: LDH Assay**

Damage in the human corneal and conjunctival epithelial cells was quantitatively evaluated by measuring the amount of soluble cytosolic enzyme LDH released from injured cells into the extracellular fluid, 10 and 30 min after exposure to the drugs, using the LDH kit, as previously described [6].

The LDH level corresponding to complete cell death was determined for each experiment by assaying sister cultures exposed to BAK 0.01% for the corresponding time. Background LDH release was determined in control cultures not exposed to drugs, and was subtracted from all experimental values. The resulting values correlated linearly with the degree of cell loss estimated at the observation of cultures under phase-contrast optics.
**Propidium Iodide Labeling**

Cell injury was assessed in conjunctival and corneal cell cultures using propidium iodide (PI), a polar dye which enters the cells only if the membrane is damaged and becomes fluorescent upon binding to DNA. PI was added to the medium with the drugs during 30 min of exposure. At the end of the 30 min, fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific, Segensworth, UK) equipped with a xenon-arc lamp, a low-power objective (×20), and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1TM; Intracellular Imaging Inc., Cincinnati, OH, USA). Five images for each treatment were analyzed using image software (ImageJ; NIH, Bethesda, USA).

**Statistical Analysis**

Data are presented as mean ± standard error of the mean (SEM) of n experiments. The statistical significance of differences in MTT, LDH release, and PI staining were analyzed using one-way analysis of variance (ANOVA) with Dunnett’s post hoc test for multiple comparisons. All statistical calculations were performed using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA, USA). A p value less than 0.05 was considered significant.

**RESULTS**

**Antimicrobial Activity**

The antimicrobial activity of Oftasecur and LP was evaluated against a panel of Gram-positive and Gram-negative bacteria and the yeast C. albicans. Results are reported in Table 1.

Oftasecur was active at dilutions ranging from 1:2 to 1:16, with the most effective antimicrobial effect against the yeast C. albicans. Oftasecur displayed bactericidal and fungicidal effect against all assayed microorganisms, including the two antibiotic-resistant S. aureus and S. epidermidis strains, at the MIC values for each organism. The LP control solution had no activity against any of the tested microorganisms.

**Time-Kill Curves**

The killing effect of Oftasecur was determined against S. epidermidis. Experiments were also performed in NaPB to evaluate spontaneous decrease in bacterial vitality in the buffer solution. At each time, the fraction of survivors was calculated as a percentage compared to the inoculum.

As shown in Fig. 1, most of the reduction of microbial vitality (65%) occurred within the first minute of exposure, and after 60 min almost 80% of the microbes died.

**Evaluation of the Effects of Oftasecur and Its Constituents on Conjunctival and Corneal Epithelial Cells Using LDH and MTT Assay**

The maximum degree of cell death was produced in our culture system by incubation with
0.01% BAK, which produced a maximum release of LDH, a quantitative cytotoxicity index.

After 10 and 30 min of incubation of conjunctival epithelial cells with drugs at different dilutions (1:1, 1:2, 1:4, and 1:32), while Oftasecur and its components displayed negligible cell death, only Biosecur resulted in toxicity at both exposure times and all dilutions (Fig. 2a, c).

No significant changes in conjunctival cell viability measured by MTT assay were detected after 10 min except for the undiluted form of LP and BB, and for Biosecur and BAK 0.01% at all dilutions (Fig. 2b); after 30 min, even the undiluted form of Oftasecur showed a significant reduction in viability (Fig. 2d).

As observed on conjunctival cells, Oftasecur, LP, and BB caused negligible corneal epithelial cell death at all dilutions, whereas Biosecur and BAK showed toxic effects at both time points and at all concentrations (Fig. 3a, c). On the other hand, in the MTT assay, we observed a reduction of cell viability with Oftasecur, LP, and BB at both exposure times but only in their undiluted forms. Biosecur, such as BAK, induced cell impairment at all experimental conditions (Fig. 3b, d).

**DISCUSSION**

Gram-positive bacteria such as S. aureus, S. epidermidis, S. pyogenes, P. aeruginosa, and E. coli as well as the yeast C. albicans are among the most common causes of ocular infections [2, 13–15]. In recent years, multidrug-resistant bacterial strains such as MRSA and MDR S. epidermidis have become a relevant issue in ophthalmology, limiting the efficacy of antibiotics in the prevention and treatment of ocular infections [2, 16]. In this context, research is focusing on drugs, such as antiseptics, with a wide antimicrobial spectrum, including MDR bacterial strains and fungi, and a good toxicity profile. Actually, the antiseptic povidone-iodine is the mainstay of prophylaxis of postsurgical infections in ophthalmology [3], but it can induce cytotoxic effects on the ocular surface, as shown by in vitro and in vivo studies, especially at high concentrations [7, 17].

The ocular spray tested in this study, Oftasecur, contains the antiseptic Biosecur, composed of citrus bioflavonoids with a wide antibacterial spectrum [9, 11]. Cormier and colleagues [9] reported the activity of Biosecur as a food-grade surface cleaner with strong inhibitory effect against *Vibrio vulnificus*, a leading cause of seafood-associated illness and death in the USA.

More recently, Vagge et al. [12], in a prospective pilot study on candidates for intraocular injections, found a significant reduction in conjunctival bacterial load after a
4-day prophylactic treatment with Oftasecur Ocular Spray, including both Gram-positive (especially *S. epidermidis*) and Gram-negative bacteria. Moreover, the product was well tolerated by patients [12].

To our knowledge, studies evaluating the in vitro antimicrobial activity of Oftasecur and its effects on conjunctival and corneal epithelial cells are lacking.

Our study showed a wide antimicrobial activity of the ocular spray against *C. albicans* and the Gram-positive and Gram-negative bacteria which are the most common cause of ocular infections, with a bactericidal and fungicidal effect at dilutions from 1:2 to 1:16.
This activity appears to be due to the presence of Biosecur inside liposomes, since no antimicrobial effect was caused by liposomes and excipients alone. Moreover, the killing activity against *S. epidermidis* was very fast: Oftasecur was able to significantly reduce by about 65% the number of alive bacteria after 1 min of incubation. The product showed antibacterial effect against drug-resistant *S. aureus* and *S. epidermidis*, suggesting its potential use as a valid antiseptic in the control of infections caused by multidrug-resistant strains.

Regarding its in vitro effects on immortalized conjunctival epithelial cells, the cytotoxicity of Oftasecur was similar to vehicle after 10 and 30 min of cell exposure, and the cell viability was significantly reduced only by Oftasecur in its undiluted form. Results were similar in
Considering also that in vivo the administered solutions are always diluted in the tear film, Oftasecur showed an optimal in vitro tolerability profile. Conversely, with Biosecur and BAK. *Veh* vehicle, *BAK* benzalkonium chloride, *LP* 1% liposomal solution without Biosecur. Data are expressed as percentage of the maximum PI staining (incubation of cells with BAK 0.01%). ***p < 0.001 vs Veh

**Fig. 4** Qualitative (a) and quantitative (b) analysis of the effects of 30 min of Oftasecur on human conjunctival epithelial cells (HConEC), evaluated using propidium iodide (PI). Inverted fluorescence microscope images (×20 magnification): the levels of fluorescence were high only with Biosecur and BAK. *Veh* vehicle, *BAK* benzalkonium chloride, *LP* 1% liposomal solution without Biosecur. Data are expressed as percentage of the maximum PI staining (incubation of cells with BAK 0.01%). ***p < 0.001 vs Veh

**Fig. 5** Qualitative (a) and quantitative (b) analysis of the effects of 30 min of Oftasecur on human corneal epithelial cells (HCE-2) evaluated using propidium iodide (PI). Inverted fluorescence microscope images (×20 magnification): the levels of fluorescence were high only with Biosecur and BAK. *Veh* vehicle, *BAK* benzalkonium chloride, *LP* 1% liposomal solution without Biosecur. Data are expressed as percentage of the maximum PI staining (incubation of cells with BAK 0.01%). ***p < 0.001 vs Veh
Biosecur induced a significant cytotoxicity and reduction of cell viability at all dilutions and at all time points. On the basis of our findings, we hypothesize that the liposomal formulation of the ocular spray tested is responsible for the reduction of the toxicity of the antiseptic Biosecur on conjunctival and corneal epithelial cells in vitro. Our hypothesis is also supported by previous studies which reported that liposomal formulations are able to reduce the toxicity of different drugs for ocular tissues [18, 19] and conjunctival and corneal cells in vitro [20]. Liposomes have in fact some advantages over most ophthalmic delivery systems: they are biodegradable, biocompatible, relatively nontoxic, and they can provide an intimate contact with the corneal surface [21]. In vivo, the liposomal formulations can improve the residence time of a drug on the ocular surface, which is usually no more than 1–3 min because of fast elimination via the nasolacrimal duct [22], thus prolonging its activity and reducing the effective dose [18].

CONCLUSION

Oftasecur showed a rapid and wide-spectrum antibacterial activity in our study, with a promising in vitro tolerability profile. The finding that the product is active at up to 1:16 dilutions, depending on the considered microorganisms, suggests that this drug can be also effective when administered in vivo, where a dilution in the tear film always occurs. Our data support the pilot clinical study by Vagge et al. [12], but further studies are necessary to confirm these results on a larger number of patients.

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Author Contributions. Conceptualization, Rita Mencucci, Emilia Ghelardi, Eleonora Favuzza, Elisa Landucci; Methodology: all authors; Formal analysis and investigation: all authors; Writing—original draft preparation and writing—review and editing: all authors; Supervision: Rita Mencucci, Emilia Ghelardi, Domenico Edoardo Pellegrini-Giampietro, Elisa Landucci. All authors read and approved the final manuscript.

Disclosures. Rita Mencucci, Emilia Ghelardi, Francesco Celandroni, Costanza Mazzantini, Alessandra Vecchione, Domenico Edoardo Pellegrini-Giampietro, Eleonora Favuzza, and Elisa Landucci declare that they have no conflict of interest.

Compliance with Ethics Guidelines. This article does not contain any study with human participants or animals performed by any of the authors. Institutional review board approval was not required at our institute for the use of immortalized cell lines.

Data Availability. Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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