RESEARCH LETTER

Eudragit E100® potentiates the bactericidal action of ofloxacin against fluoroquinolone-resistant *Pseudomonas aeruginosa*

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Abstract

We report the enhanced bactericidal activity of ofloxacin in drug-containing Eudragit E100® dispersions (EuCl-OFX) against *Pseudomonas aeruginosa* and the effect of the cationic polymer on bacterial membrane. Organisms treated with EuCl-OFX showed changes in cell morphology, altered outer membrane (OM) and cytoplasm with low electrodensity areas. Zeta potential of bacterial surface was shifted to positive. Sensitization to lytic agents was also observed. A profound effect on bacterial size, granularity and membrane depolarization was found by flow cytometry. Cultures exposed to drug-free polymer also showed some damaged bacterial membranes, but there was no significant cell death. Inhibition of *P. aeruginosa* by EuCl-OFX may involve surface effect and, to some extent, permeation effect. The cationic polymer act to mitigate the electronegativity of cell surface in the process of disorganizing the OM, rendering it more permeable to antibiotic. In addition, cytoplasmic membrane depolarization turns bacterial cell more vulnerable. The effects on membranes combined with the mechanism of action of quinolone explain the improved bactericidal action exhibited by EuCl-OFX. The behavior described for Eudragit E100® against *P. aeruginosa* may be a useful tool to broaden the spectrum of antibiotics whose clinical use is limited by the impermeability of the bacterial OM.

Introduction

Infections caused by *Pseudomonas aeruginosa* are difficult to treat due to the intrinsic resistance of this microorganism to multiple classes of antimicrobial agents and to the ability to acquire induced resistance during therapy (Aloush *et al.*, *2006;* Bertino, 2009; Giamarellou, 2010). Strategies devised to reduce microbial multiresistance include control measures for the use of antibiotics, detection of genetic resistance mechanisms, a search for new synthetic or natural substances with antimicrobial activity or those contributing to enhancing the action of known antibiotics as well as the development of new strategies of drug delivery (Wright, 2010; Moellering, 2011).

The resistance of *P. aeruginosa* to antibiotics is primarily attributed to reduced outer membrane (OM) permeability (Nikaido, 1989; Hancock, 1998; Lambert, 2002). Strategies to minimize the low OM-permeability of *P. aeruginosa* could significantly contribute to the success of therapy for infections with this organism. Several reports describe the effect of cationic peptides as antimicrobial agents or diverse agents as detergents or polymers used in attempts to alter the OM-permeability of Gram-negative bacteria (Mugabe *et al.*, 2006; Dillen *et al.*, 2008; Tin *et al.*, 2009; Romero *et al.*, 2010).

Most bacteria carry a net negative surface charge. Therefore, some interaction with positively charged materials is expected due to electrostatic attraction forces.

Eudragit E100® (Eu) is a cationic polymer based on dimethylaminoethyl methacrylate and other neutral methacrylic acid esters used in several applications in the pharmaceutical field (Rowe *et al.*, 2006). The ionic interaction between protonated amino groups of Eu neutralized with acidic drugs and hydrochloric acid yields water-soluble complexes (Quinteros *et al.*, 2008).

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Formerly, pharmaceutical excipients were considered to be inert substances devoid of biological action. However, several reports indicate that excipients not only determine the physicochemical properties of a dosage form but may also confer new and unexpected biological properties. In particular, eukaryotic membrane destabilizing properties and the reversible permeation enhancing effect have been reported for Eudragit E100® (Alasino *et al.*, 2005; Grube *et al.*, 2008). However, there are no data on the microbicidal activity or interaction with bacteria.

Ofloxacin is a broad-spectrum fluoroquinolone selected in this work to be loaded on Eudragit E100® dispersions.

The aim of this study was to compare the performance of ofloxacin-containing polymer dispersions (EuCl-OFX) with free ofloxacin solution against fluoroquinolone-resistant *P. aeruginosa* and to investigate the effect of cationic polymer in the bacterial membrane.

Materials and methods

Preparation of ofloxacin-containing Eudragit dispersions

The equivalents of amino groups per gram of Eudragit E100® (3.10×10^{-3}) were determined by acid-base titration. Ofloxacin-containing Eudragit dispersions were prepared according to previous guidelines (Quinteros *et al.*, 2008) with slight modifications. Briefly, Eu was dissolved in acetone and 1.0 N HCl was added to neutralize 50% of the amino groups to overcome solubility limitations. The solvent was evaporated at room temperature. EuCl (fine powder) was dissolved in a minimum amount of water, ofloxacin was added to neutralize 20% of the amino groups of the polymer and the volume was adjusted to produce the final stock solution (4.52 mg mL^{-1} ofloxacin in EuCl-OFX); concentration selected to avoid unwanted side effects described for ofloxacin ophthalmic formulations containing greater than 5 mg mL^{-1} (Gurny & Felt, 2003).

Electrokinetic potential measurements (ζ)

Electrokinetic potentials were measured by Electrophoretic light scattering, using Delsa Nano C instrument (Beckman Coulter, Japan) equipped with a 658-nm laser diode and temperature controller. The EuCl-OFX aqueous dispersion measurements were performed in triplicate at a scattering angle of 165° at 25 °C, allowing the instrument to automatically optimize the signal intensity of the sample. The zeta potential of bacterial suspensions of *P. aeruginosa* FQ-R1 ($\approx 10^7$ CFU mL^{-1}) in deionized water or EuCl-OFX-treated for 10 min was measured at a scattering angle of 90° at 37 °C. Bacterial suspensions were placed into the flow cell and zeta potential measurements were performed at least four times for individual samples.

Bacterial strains

Clinical isolates of *P. aeruginosa* used in this study are resistant to fluoroquinolone (Table 1). Bacteria were stored at −20 °C in Trypticase Soy Broth supplemented with 10% glycerol. Fresh cultures were maintained in water at room temperature.

Bactericidal profiles

Killing-curve studies were performed in saline because Eudragit E100® partially precipitated in the culture medium during the long incubation period.

Overnight culture in Müeller–Hinton broth were adjusted to a bacterial concentration of approximately 10^8 cells mL^{-1} and incubated in the presence of ofloxacin in EuCl-OFX or free solution, assessing a range of concentrations from sub- to several multiples of each organism's ofloxacin minimum inhibitory concentration (MIC).

Bacterial suspensions treated with drug-free polymer (EuCl) were also evaluated at identical concentrations of EuCl to those contained in EuCl-OFX dilutions, ranging from 150 to 9600 μg mL^{-1}. A tube without treatment was used as a growth control. The cultures were incubated at 37 °C and sampled periodically up to 24 h. The number of viable cells was determined by subculturing the cells on Mueller–Hinton agar plates in duplicate for 24 h. Each time-dependent killing experiment was performed on three independent occasions and the data presented are the average of all values obtained.

Transmission electron microscopy

Pseudomonas aeruginosa overnight culture was suspended to approximately 40 mg (wet weight) mL^{-1} in 50 mM

Table 1. *In vitro* susceptibility to fluoroquinolones of *Pseudomonas aeruginosa* isolates used in this study*

	MIC (μg mL^{-1})*			
Strain	Ofloxacin	Ciprofloxacin	Norfloxacin	Levofloxacin
P. aeruginosa ATCC 27853	2–4	0.5	8	2
P. aeruginosa FQ-R	32	4–8	32	16
P. aeruginosa FQ-R1	128	64–128	256	32–64
P. aeruginosa FQ-R2	256	128	256	128

FQ-R, fluoroquinolone-resistant.

*Data from previous work in our laboratory (Romero *et al.*, 2010).

phosphate buffer (pH 7.4). Aliquots were treated with EuCl-OFX (ofloxacin concentration 200 μg mL^{-1}) and incubated for 3 h at 37 °C. Aliquots 500 μL were centrifuged (3200 ***g*** for 5 min). The pellet was washed twice in phosphate buffer and fixed in 4% formaldehyde and 2% glutaraldehyde mixture in cacodylate buffer 0.1 M (2 h at room temperature). Bacteria were washed three times with cacodylate buffer and postfixed in 1% osmium tetraoxide in distilled water for 1–2 h at room temperature. The cells were dehydrated with gradients of acetone and embedded in Araldite epoxy resin and polymerized at 60 °C for 24 h. Thin-sections (80–100 nm width) were obtained using a Jeol Jum-7 ultramicrotome. The samples stained with uranyl acetate in alcoholic solution (2 min) and lead citrate (2 min) were analyzed using a LEO 906 E transmission electron microscope at an operating voltage of 80 kV. Images were captured with a MegaView III camera.

Additional aliquots of bacterial suspension were treated with EuCl and ofloxacin or supplemented with phosphate buffer (control).

Sensitization to lytic action of lysozyme and detergents

Bacteria grown overnight were collected, suspended in saline and suspensions adjusted to an absorbance of 0.3. Cells were exposed to 32 μg mL^{-1} of drug in EuCl-OFX for 10 min at 37 °C. Following centrifugation, bacterial cells were re-suspended in saline containing 10 μg mL^{-1} lysozyme, 1%Triton X-100 or 0.1 and 1% SDS. Suspensions were incubated for an additional 4 min at 37 °C and cell lysis was measured as a decrease in optical density at 405 nm. Results were expressed as the percentage of controls. Strong lysis is thus indicated by a low percentage of $OD_{405\,nm}$. Polymyxin B (100 μg mL^{-1}) was used as a positive control.

Flow cytometry

Culture overnight was adjusted in saline to an absorbance of 0.3 at 625 nm. Aliquots were exposed to EuCl-OFX (drug concentration range from 8 to 512 μg mL^{-1}), ofloxacin, EuCl or saline alone (control). The mixtures were incubated at 37 °C and samples taken after 1, 3, 6 and 24 h. Aliquots were centrifuged (3200 ***g*** for 2 min) and washed with saline. $DiBAC_4$ was dissolved in 70% ethanol (1 mg mL^{-1}) and further diluted in deionized water (5 μg mL^{-1}). Twenty microlitres were added to 180-μL aliquots of the recovering cultures (final dye concentration 0.5 μg mL^{-1}). After 5 min in the dark at room temperature, mixtures were acquired on a BD FACS Canto II (BD Biosciences, CA) equipped with a 488-nm argon-ion laser. Forward-scatter (FSC-A), side-scatter (SSC-A) and fluorescence signals were collected in logarithmic scale. At least 10 000 events were recorded for each sample, and all experiments were conducted in duplicate on separate days.

Aliquots of cultures exposed 24 h to EuCl-OFX, ofloxacin and EuCl were streaked on solid culture medium and incubated overnight.

Results

Ofloxacin-containing Eudragit aqueous dispersions are physically stable, possess a positive electrokinetic potential (24 mV) and pH values ranged 6.2–6.4.

Figure 1a–e shows the bactericidal properties exhibited by EuCl-OFX and ofloxacin free solution at different multiples of ofloxacin MIC for *P. aeruginosa* FQ-R1. Each plot also presents the effect of drug-free polymer at concentrations equivalent to those present in EuCl-OFX.

EuCl-OFX tended to kill *P. aeruginosa* FQ-R1 very rapidly, achieving a 3 $\log_{10}$ decrease between 1 and 3 h at ¼ × MIC ofloxacin (32 μg mL^{-1}) (Fig. 1a), whereas > 6 h of exposure was required for ofloxacin.

Eradication was achieved within the first hour of assay after exposure to EuCl-OFX at 1024 μg mL^{-1} (8 × MIC ofloxacin, Fig. 1e), whereas the ofloxacin free solution did not yield bacterial eradication in the entire range of drug concentrations evaluated. At longer exposure times, EuCl-OFX eradicated at drug concentrations 4–16 times lower than those required with ofloxacin. For instance, after 3 h exposure to EuCl-OFX, eradication of *P. aeruginosa* FQ-R1 was observed at ofloxacin concentrations of 256 μg mL^{-1} (2 × MIC, Fig. 1c) and 1024 μg mL^{-1} (8 × MIC, Fig. 1e) were required for free ofloxacin. Accordingly, 32 μg mL^{-1} of drug in EuCl-OFX yielded a complete bacterial eradication after 24 h (Fig. 1a) in comparison with 512 μg mL^{-1} of free ofloxacin (Fig. 1d).

Drug-free EuCl exhibited bacteriostatic or slightly bactericidal action depending on the concentration, time or strain considered with regrowth seen at longer exposure times.

The enhanced performance described above for EuCl-OFX was also observed against *P. aeruginosa* FQ-R2 (data not shown), exhibiting a bactericidal effect at sub-MIC ofloxacin concentrations in the early hours of the experiment. Eradication was achieved with EuCl-OFX at 2048 μg mL^{-1} (8 × MIC ofloxacin for *P. aeruginosa* FQ-R2) within the first hour of assay.

After brief exposure to EuCl-OFX, the zeta potential of *P. aeruginosa* FQ-R1 was modified in value and sign (from −26.8 to 14.5 mV). The cationic nature of Eudragit is the key factor contributing to its interaction with the negatively charged microbial cell surface. The binding neutralizes and even reverse the surface charge of the

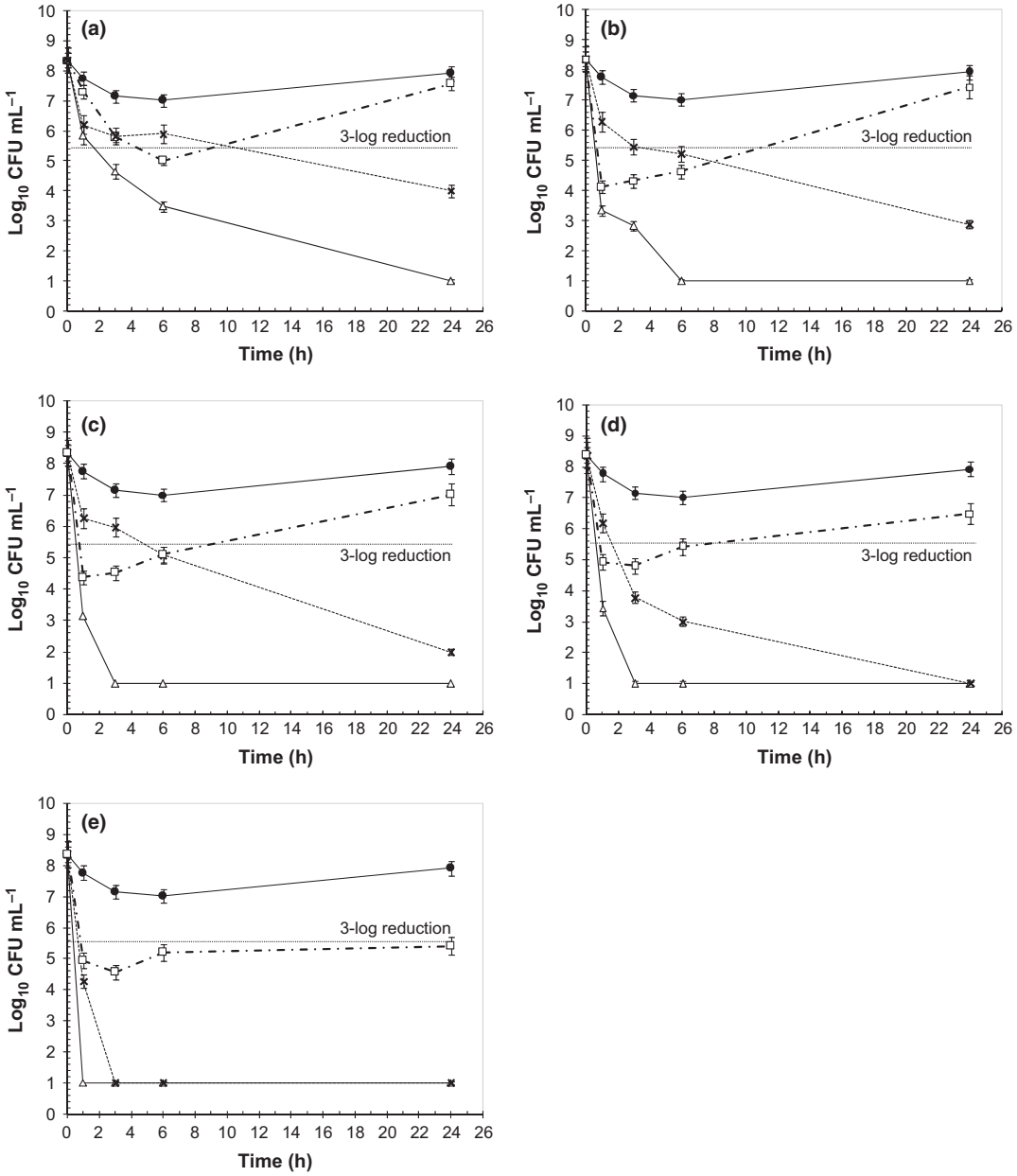

Fig. 1. Time-kill curves showing the effects of EuCl-OFX (Δ, solid line) and ofloxacin solution (×, dotted line) at different multiple ofloxacin MICs against *Pseudomonas aeruginosa* FQ-R1. (a) ¼ × MIC; (b) 1 × MIC; (c) 2 × MIC; (d) 4 × MIC; (e) 8 × MIC. Each plot also includes untreated control (●, solid line) and the effect of free ofloxacin polymer (EuCl; □, dash-dot line) at concentrations equivalent to those present in EuCl-OFX.

bacteria. At this stage, the change is reversible. Cultures under the action of OFX showed no effect, in agreement with that previously reported for *Escherichia coli* with ciprofloxacin (Dealler, 1991).

Most of the cells treated with EuCl-OFX for 3 h revealed alterations in their shape, cytoplasmic density and irregularities in bacterial cell wall which could affect the functionality of the normal cell membrane (Fig. 2a). Although ofloxacin-treated cells showed slight changes in cytoplasmic electrodensity (*, Fig. 2b), the bacterial membranes were still unaltered and cell morphology was preserved. Untreated controls show normal appearance (Fig. 2d).

Exposure of *P. aeruginosa* FQ-R1 to EuCl-OFX before adding detergent or lysozyme resulted in lysis of 5.6 ± 6.8% of cells (data not shown). Similarly, treatment with polymyxin B resulted in lysis of 8.5 ± 4.6% of cells. Bacteria culture was weakly sensitized by EuCl-OFX to Triton X-100 and lysozyme, but strongly sensitized to SDS (Table 2). Bacteria cell lysis by lytic agents following

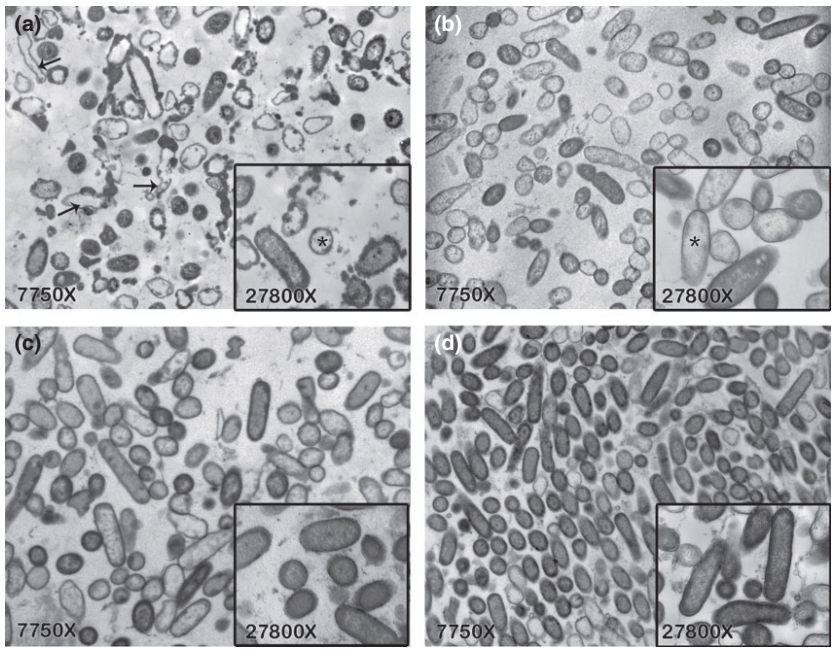

Fig. 2. Transmission electron microphotographs of *Pseudomonas aeruginosa* FQ-R1 showing the loss of normal bacterial shape after treatment with (a) EuCl-OFX (→). (b) Cytoplasmic low density (*) is observed after ofloxacin treatment. (c) Drug-free polymer (EuCl). (d) Untreated control.

polymyxin treatment, a known OM-disorganizing agent, did not differ significantly. By contrast, cultures treated with ofloxacin did not differ with the control.

$DiBAC_4$ is fluorescent probe voltage sensitivity that enters depolarized cells (Müeller & Straüber, 2010), used to estimate damage of membrane potential in *P. aeruginosa* treated with EuCl-OFX. Figure 3 presents the effects of increasing concentrations of EuCl-OFX, drug-free polymer (EuCl) and free ofloxacin on the membrane potential for three isolates of *P. aeruginosa*. The negative controls showed the minimum relative fluorescence intensity (Fig. 3a, e and i). Accordingly, we considered the M1 range to be undamaged cells showing no significant depolarization of cytoplasmic membrane, and the M2 range to be damaged cells. The cell proportions exhibiting dye-associated fluorescence (M2) are expressed as percentages.

The results indicate a rapid depolarization of cells treated with EuCl-OFX. After 1 h exposure, $DiBAC_4$-associated fluorescence increases in intensity between 1 and 3 log orders, depending on the concentration and the strain analyzed. The percentage of fluorescent *P. aeruginosa* FQ-R1 (Fig. 3b–d) and -R2 (Fig. 3f–h) cells ranged from 87% to 100% and a concentration-dependent effect was found. This effect was more noticeable in the behavior of *P. aeruginosa* FQ-R (Fig. 3j–l), experiments in which drug concentrations and polymer were lower than those used for *P. aeruginosa* FQ-R1 and -R2. In those cases, the proportion of fluorescent bacteria was only 74% when exposed for 1 h at the lowest concentration of EuCl-OFX tested without reaching 90% at the highest concentration.

Table 2. Sensitization of *Pseudomonas aeruginosa* FQ-R1 to lytic action of lysozyme and detergents

	Relative turbidity (%)				
Lytic agent	Control	EuCl-OFX	EuCl	Ofloxacin	Polymyxin
Lysozyme	92 ± 3	83 ± 11	82 ± 15	93 ± 5	73 ± 10
SDS 1%	52 ± 4	9 ± 1	17 ± 8	53 ± 5	15 ± 14
SDS 0.1%	100 ± 6	8.6 ± 6	16 ± 3	95.5 ± 9.2	14.6 ± 4.7
Triton X-100	88 ± 6	72 ± 1	77 ± 2	92 ± 5	88 ± 6

In contrast, the percentages of fluorescing bacteria exposed to ofloxacin for 1 h were < 2% and are similar to those obtained with the control culture.

No changes were observed for any of the drug concentrations tested when the time of exposure was prolonged up to 24 h.

Membrane depolarization observed after exposure to EuCl-OFX was similar to that exhibited by cultures treated with drug-free polymer (EuCl). Therefore, the effect on the membrane potential could be attributed to the concentration of cationic polymer in the EuCl-OFX. Nevertheless, no survivor was recovered on solid culture medium after 24 h exposure to EuCl-OFX, whereas electrostatically depolarized cells from cultures exposed to EuCl grew freely on agar plates. This shows that the depolarization indicates decreased cell functionality but

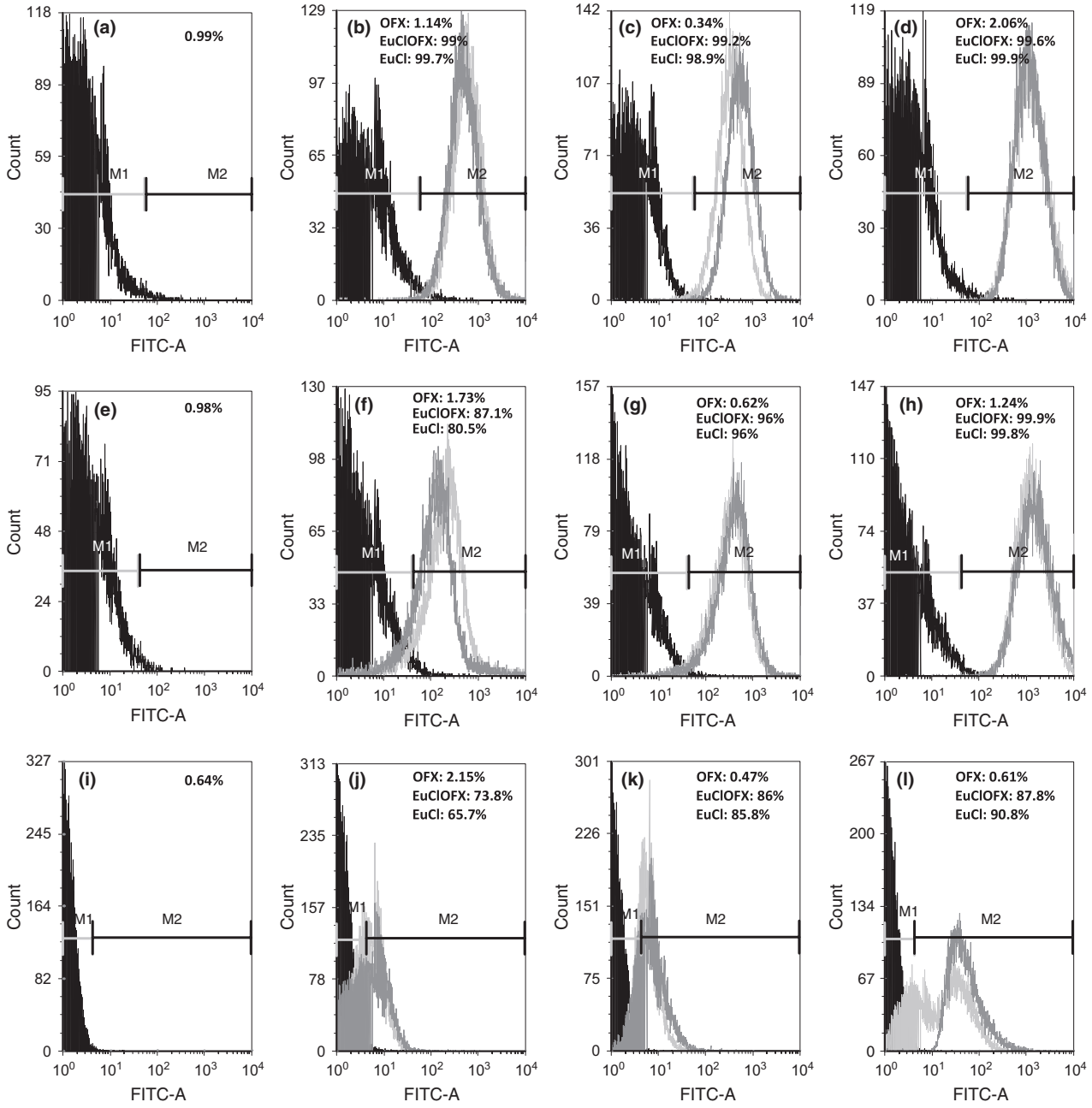

Fig. 3. Membrane depolarization ($DIBAC_4$ fluorescence) in three isolates of *Pseudomonas aeruginosa* after 1 h exposure to EuCl-OFX (light gray line), drug-free polymer (EuCl; gray line) and free ofloxacin solution (black line). Drug/polymer concentrations from left to right: (a, e) untreated control; (b, f) 32/146 μg mL^{-1}; (c, g) 128/584 μg mL^{-1}; (d, h) 512/2336 μg mL^{-1} against *P. aeruginosa* FQ-R1 (upper panels) and *P. aeruginosa* FQ-R2 (middle panels). Drug/polymer concentrations for *P. aeruginosa* FQ-R (lower panels): (i) untreated control; (j) 8/36.5 μg mL^{-1}; (k) 16/73 μg mL^{-1}; (l) 32/146 μg mL^{-1}. Percentages indicate proportion of depolarized cells (M2).

certainly not cell death. These results are consistent with those shown in Fig. 1.

Histograms in Fig. 4 show changes in size (FSC-A) and granularity (refractory index, SSC-A) of *P. aeruginosa* FQ-R1 after 1 h of exposure to EuCl-OFX. A concentration-dependent shift in both parameters was observed. A new population of events exhibiting a smaller forward scatter appeared and the mean intensity of FSC-A was reduced compared with free ofloxacin (a–d). Although this behavior was seen at all concentrations assayed, a heterogeneous bacterial size distribution was more evident at high concentrations (Fig. 4d).

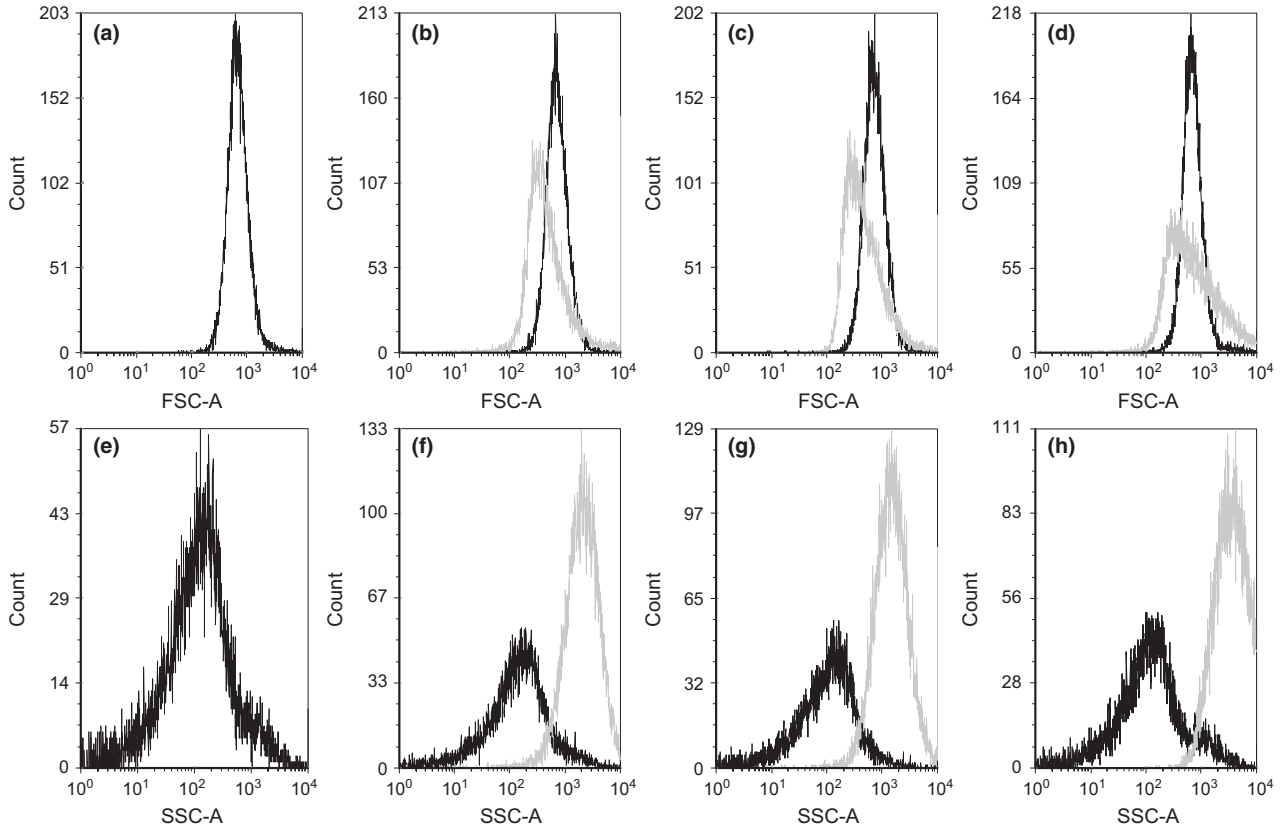

Fig. 4. Flow cytometric histograms comparing the size distributions (FSC-A; upper panels) and granularity (SSC-A; lower panels) from cultures of *Pseudomonas aeruginosa* FQ-R1 at 1 h after exposure to EuCl-OFX (gray lines) and OFX (black lines). Drug/polymer concentrations: (a, e) untreated control; (b, f) 32/146 μg mL^{-1}; (c, g) 128/584 μg mL^{-1}; (d, h) 512/2336 μg mL^{-1}.

The granularity histograms (Fig. 4e–h) clearly show a well defined population of events with a much higher side scatter in cultures treated with EuCl-OFX, exhibiting more than 1 log order increase in SSC-A mean values and a concentration-dependent effect. Only a small number of events remained in the area occupied by the control population. Cultures exposed to drug-free polymer (EuCl) exhibit similar behavior to those exposed to EuCl-OFX (data not shown). By contrast, free ofloxacin did not induce any measurable change in FSC-A or SSC-A over the wide range of concentrations evaluated in comparison with the control, even after longer exposure times (up to 24 h).

The same effect on the granularity and size of bacterial cells described for *P. aeruginosa* FQ-R1 was observed in experiments testing *P. aeruginosa* FQ-R and -R2 (data not shown).

Discussion

Quinolones are concentration-dependent antimicrobial agents and the optimal bactericidal concentrations are close to eight times their MICs; however, EuCl-OFX exhibited higher and faster bactericidal action than free ofloxacin from sub-MIC concentrations of each strain and inoculum eradication at prolonged time acts as a potentiating agent for fluoroquinolone.

Fluoroquinolones must enter the cell to become effective; therefore, the properties of the cell surface properties play an essential role in the determination of antimicrobial resistance. Electrostatic interactions between negatively charged bacteria and EuCl-OFX (positive zeta potential) put them in touch quickly and reverse the bacterial surface charge. EuCl-OFX has a strong OM-permeabilizing activity at concentrations below the levels needed to achieve eradication of inocula after brief exposure (sub-MIC concentrations of OFX). Although there are reports of OM-permeabilizing action for some fluoroquinolones, it arises as a side effect and occurs after prolonged exposure to supra-MIC concentrations (Chapman & Georgopapadakou, 1988; Vaara, 1992; Mason *et al.*, 1995). Moreover, a previous report showing that ofloxacin does not sensitize *P. aeruginosa* to hydrophobic antibiotics (Vaara, 1992) contributes to our results, attributing the observed effect to the action of cationic polymer.

EuCl-OFX interacts with both bacterial cell membranes. In addition to the OM permeabilization, EuCl-OFX causes concentration-dependent depolarization of cytoplasmic membrane in *P. aeruginosa* cells. The alterations in the bacterial envelopes are reflected in the changes observed in size and granularity of the bacterial cell.

Drug-free polymer exhibited bacteriostatic or weakly bactericidal effect after a short exposure time and subsequently recovered. According to the performance of other known polycationic permeabilizers (Vaara, 1992), our results indicate that, to a large extent and despite being a powerful permeabilizer, EuCl does not kill *P. aeruginosa*. This lack of correlation between cytoplasmic membrane depolarization and bacterial cell lethality was also described for cationic antibacterial peptides (Zhang *et al.*, 2000).

The inhibition of *P. aeruginosa* growth by EuCl-OFX may involve surface effect and, to some extent, permeation effect. The cationic polymer would mitigate the electronegativity of cell surface in the process of disorganizing the OM, rendering it permeable to antibiotic. In addition, cytoplasmic membrane depolarization turns bacterial cell more vulnerable. Therefore, the bactericidal action exhibited by EuCl-OFX is derived from a mechanism combining OM-permeabilization and bacterial membrane depolarization coupled with the action of fluoroquinolones on intracellular target.

To our knowledge, this is the first study on the interaction of Eudragit E100® with bacterial cells. Although Eudragit E100® is not bactericidal in itself, the ability to alter the OM of *P. aeruginosa* and induce changes in membrane potential extends the applicability of this polymer as a vehicle for drug delivery into cells or as an adjuvant or potentiator for fluoroquinolones in topical pharmaceutical preparations. EuCl-OFX exhibits pH within the acceptable range to be administered as an ophthalmic formulation. The cationic polymers may interact with the negatively charged layer of mucus in the eye surface and induce a significant increase in the precorneal residence time of the preparations (Dillen *et al.*, 2006). In addition, recent studies indicating Eudragit E100® is well tolerated in rabbit eyes (Quinteros, 2010) support the potential use of EuCl-OFX in the design of an ophthalmic formulation.

Furthermore, the potentiator effect described for Eudragit E100® against *P. aeruginosa* may be a useful tool to broaden the spectrum of antibiotics whose clinical use is limited by the impermeability of the bacterial OM.

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