J Membrane Biol (2011) 241:117–125
DOI 10.1007/s00232-011-9368-4

New Insights into the Translocation Route of Enrofloxacin and Its Metalloantibiotics

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Received: 3 February 2011 / Accepted: 27 April 2011 / Published online: 17 May 2011

Abstract Probing drug/lipid interactions at the molecular level represents an important challenge in pharmaceutical research, drug discovery and membrane biophysics. Previous studies showed that enrofloxacin metalloantibiotic has potential as an antimicrobial agent candidate, since it exhibits antimicrobial effect comparable to that of free enrofloxacin but a different translocation route. These differences in uptake mechanism can be paramount in counteracting bacterial resistance. In view of lipids role in bacterial drug uptake, the interaction of these compounds with different *Escherichia coli* model membranes were studied by fluorescence spectroscopy. Partition coefficients determined showed that lipid/antibiotic interactions were sensitive to liposomes composition and that the metalloantibiotic had a higher partition than free enrofloxacin. These results corroborate the different mechanism of entry proposed and can be rationalized on the basis that an electrostatic interaction between the metalloantibiotic positively charged species, present at physiological pH, and the lipids negatively charged head groups clearly promotes the lipid/antimicrobial association.

Keywords Antimicrobial · Cardiolipin · *Escherichia coli* membrane · Enrofloxacin · Fluorescence spectroscopy · Metalloantibiotic

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The introduction of fluoroquinolones (FQ), such as enrofloxacin (Erx), more than 20 years ago offered clinicians a range of antimicrobial agents that have a broad spectrum of activity (Alvarez et al. 2008) together with the capacity of acting against extracellular and intracellular infections (Martinez et al. 2006; Van Bambeke et al. 2005; Seral et al. 2005). However, the extensive therapeutic use of FQ antimicrobial agents in food animal production, particularly in poultry, has become a concern for public health (Martinez et al. 2006; Mitchell 2006). It is known that there is a worldwide problem with the increase of pathogenic bacteria that are multiresistant to commercially available antibiotics since the number of novel antibiotics on the market is declining and several strategies to retrieve control on bacterial infections are being developed (Hooper 1999; Hopkins et al. 2005). Meanwhile, the need for the design of new antibacterial agents has pushed forward the concept that metal complexes as novel derivatives of FQ, also called metalloantibiotics, can play an important role in the field (Efthimiadou et al. 2007).

Erx, a second generation FQ that exhibit not only an increased antibacterial activity against the Gram-negative bacteria but also present activity against certain Gram-positive bacteria, was the first approved FQ for use in animals, but the prevalence of Erx resistance has been known since 1996, especially regarding *Escherichia coli* isolates (Aarestrup 2006). A recent study showed that the ternary complex copper (II)/1,10-phenantroline (Phen)/Erx, seems worth pursing as a possible antimicrobial agent candidate (metalloantibiotic) because it has an antimicrobial effect comparable to that of free Erx but a different translocation route (Pate et al. 2010; Saraiva et al. 2010).

In Gram-negative bacteria, the external membrane is the major obstacle to antibiotics entrance and there are different ways known on how the antibiotic can reach the

intracellular space, namely by the transmembrane proteins, porins and/or by simple diffusion, a feature that is directly related to the antibiotics hydro/lipophilic characteristics (Bensikaddour et al. 2008; Valeur 2002; Bryan and Bedard 1991; Fresta et al. 2002; Lindner et al. 2002; Rodrigues et al. 2002). Several antibiotic molecules (some quinolones, antimicrobial peptides, etc.) pass the membrane barrier and act on cytosolic targets (Lohner and Blondelle 2005; Lohner 2009), which promotes the study of antibiotic membrane interactions not only to try to understand their mechanism of action but also to try to understand and counteract bacterial resistance (Bensikaddour et al. 2008; Cheng et al. 2011; Pagès et al. 2008).

A current trend in antibiotic research is targeting bacterial membranes to destabilize them or to induce the formation of membrane domains (Bensikaddour et al. 2008; Lopes et al. 2004; Arouria et al. 2009). Direct attack on bacterial membranes is thought to be a way to counteract bacterial resistance mechanisms, which are mostly based on intracellular adaptations (Pagès et al. 2008; Hamill et al. 2008; Epand and Epand 2009a, b; Tossi et al. 2000; Bhunia et al. 2010). This is most likely due to the differences in the lipid composition of membrane types. While the outer leaflet of mammalian plasma membranes almost exclusively consists of neutral phospholipids, the bacterial one has a high content of negatively charged phospholipids, which is supposed to be a predominant factor for the affinity of several kinds of antibacterial molecules (Cheng et al. 2011; Maniti et al. 2010; Dennison et al. 2008; Epand and Epand 2009a, b; Yeagle 1987; Epand and Epand 2011). Bacterial cell membranes have high contents of negatively charged lipids such as phosphatidylglycerol (PG) and cardiolipin (CL). The level of CL is known to change under certain environmental conditions and was described to concentrate in specific membrane locations (Hoch 1992; Mileykovskaya 2007; Schlame 2008). Therefore, it is becoming evident that is of common interest not only to study antibiotic membrane interaction but also is fundamental to use a good model membrane system of bacterial membrane. To mimic membrane systems several properties should be taken in consideration, such as the following: lipid thermotropic behavior, membrane surface lipid net charge, lipid packing density, and lipid molecular shape (Yeagle 1987; Lopes et al. 2010).

In this work two lipidic model systems that mimic *E. coli* membrane, a Gram-negative bacteria known to have bacterial resistance to Erx, were used. The simple binary system phosphatidylethanolamine:phosphatidylglycerol (PE:PG), namely 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), is widely used as model systems for the *E. coli* membrane (Murzyn et al. 2005; Lohner et al. 2001; Pozo Navas et al. 2005); nevertheless, recent work has shown that a ternary system composed of POPE:POPG:CL has a thermotropic behavior and a membrane surface lipid net charge closer to the one described for *E. coli* membrane extracts (Lopes et al. 2010) that has in its membrane lipidic composition different kinds of phospholipids in which the most abundant components are PE (57%), PG (15%) and CL (10%), and other unidentified lipids (18%) (Yeagle 1987; Avanti Polar Lipids, http://www.avantilipids.com/).

Because CL seems to be particularly prominent in several bacteria and its importance to membrane properties has been highlighted in several recent papers (Romantsov et al. 2009; Lewis and McElhaney 2009), it is significant to understand the differences or similarities of these to mimetic systems in what concerned antibiotics interaction. In this work the partition coefficients of Erx and its metalloantibiotic, were determined in the two mimetic systems mentioned above and in *E. coli* polar and total lipid extracts. The results obtained showed that the insertion of CL improved the similarities between mimetic systems and *E. coli* total extract, emphasizing the importance of negatively charged lipids to mimic *E. coli* membrane. Moreover, comparing the behavior of Erx with that observed for the metalloantibiotic it seems plausible the hypothesis, which has previously been put forward, that microorganisms resistant to pure fluoroquinolones could be sensitive to their metalloantibiotic derivatives (Saraiva et al. 2010).

Materials and Methods

All compounds were used as received: enrofloxacin (1-cyclopropyl-7-(4-ethyl-1-piperizaninyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolone carboxilic acid) (Erx) and 1, 10-phenanthroline (Phen) N-(2-hydroxyethyl) piperazine-N′-ethanesulfonic acid (HEPES) were from Sigma-Aldrich (grade pro analysis). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), CL (natural extract from *E. coli*), *E. coli* total and polar extract lipids were from Avanti Polar Lipids. All other chemicals were from Merck. All solutions were prepared with 10 mM HEPES buffer (0.1 M NaCl; pH 7.4) using Millipore water.

Metalloantibiotic Preparation

On the basis of the formation constants determined, a 10^{-5} M aqueous solution of the copper–Erx–Phen complex was prepared by mixing the components in stoichiometric proportions of (1:1:1). At pH 7.4 there are two species of complex formed, with formation percentages of 37% (CuHErxPhen) and 42% (CuErxPhen), as reported by Saraiva et al. (2010).

Liposome Preparation

Chloroform solutions (*E. coli* lipids, POPE:POPG and POPE:POPG:CL mixtures) containing the appropriate amount of lipids were dried under a stream of oxygen-free argon in a conical tube. The thin film obtained was kept under high vacuum for more than 3 h to remove organic solvent traces. Multilamellar vesicles (MLVs) were obtained after redispersion of the film in 10 mM HEPES buffer (0.1 M NaCl, pH 7.4) and vortexed above the phase transition temperature (Lopes et al. 2010). Frozen and thawed MLVs were obtained by repeating five times the following cycle: freezing the vesicles in liquid nitrogen and thawing the sample in a water bath. Suspensions of MLVs were then extruded 10 times, on a Lipex Biomembranes extruder attached to a circulating water bath, through polycarbonate filters (100 nm) to produce large unilamellar vesicles (LUVs) for POPE:POPG mixtures (with or without CL) and extruded 2, 5, and 10 times, through polycarbonate filters of 600, 200, and 100 nm, respectively, for *E. coli* total and polar lipid extract. All lipid ratios presented in this work are molar lipid ratios.

Dynamic Light Scattering

The size distribution of the liposomes, for the lipidic systems understudy, was determined by dynamic light-scattering (DLS) analysis using a Zeta Sizer Nano ZS dynamic light-scattering instrument (Malvern, UK).

Spectroscopic Measurements

Fluorescence measurements were performed on Varian Cary Eclipse spectrofluorometer equipped with a Signal Cell Peltier accessory as temperature controller. All the spectra were recorded at 37°C, with a slit width of 5 nm, wavelength of excitation of 272 nm, scan rate of 120 nm/min, data range of 1 nm and in the range from 300 to 530 nm for emission.

Partition Studies

Small aliquots (μL) of LUVs (at 37°C) of the system understudy were successively added to an aqueous solutions of Erx (8.7×10^{-6} M), or of its metalloantibiotic (9.0×10^{-6} M), to achieve final lipid concentrations in the 0–1.6 mM range. The mixtures were left to incubated at 37°C for 5 min, after which the emission spectra was traced.

Partition Coefficient Determination

Erx (and its metalloantibiotic) has fluorescent properties that enable partition coefficient determination by fluorescence spectroscopy without using external probes. The partition coefficients were calculated by the emission fluorescence spectral area integration, to which was subtracted the integrated area of the fluorescence spectra of Erx/metalloantibiotic in aqueous solution. Fluorescence spectra of the Erx solution, to which were added increasing volumes of liposomes suspension, were traced and corrected for the dilution effect (Lakowicz 1999).

The partition coefficient (K_p) of any compound between vesicle suspensions and aqueous solution is defined as:

$$K_p = \frac{\left(\frac{C_l}{C_t}\right)\Big/[L]}{\left(\frac{C_w}{C_t}\right)\Big/[W]} \quad (1)$$

where C is the drug molar concentration, the subscripts l and w indicate drugs in lipid and in aqueous media, respectively, and $[L]$ and $[W]$ represent lipid and water molar concentrations. Partition coefficients were determined without phase separation of drug/liposome suspensions (Rodrigues et al. 2002). Fluorescence data were analyzed using the following equation (Rodrigues et al. 2002; Coutinho and Prieto 1995; Santos et al. 2003):

$$\Delta I = \frac{\Delta I_{\max} K_p [L]}{[W] + K_p [L]} \quad (2)$$

where I is the fluorescence intensity (FI) of the antibiotic/metalloantibiotic measured in the presence of lipid vesicles and I_0 is the intensity measured in its absence. $\Delta I_{\max} = I_\infty - I_0$, where I_∞ is the limiting value of I. Spectral changes resulting from antibiotic–lipid interactions can be used to obtain the partition coefficient because the background signals from liposome scattering do not interfere under the experimental conditions used.

Results and Discussion

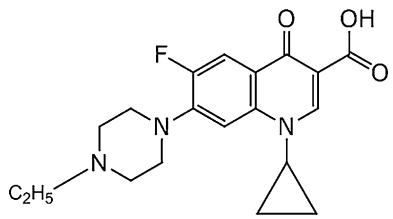
Erx has two relevant ionizable functional groups: a carboxylic acid group at position 3 of the quinolone ring and a basic piperazinyl group at position 7 (Scheme 1) with $pKa_1 = 6.17 \pm 0.01$ and $pKa_2 = 7.72 \pm 0.01$, respectively (Saraiva et al. 2010). Despite the rough similarity observed between these values for Erx and those obtained for other analogous fluoroquinolones, these small differences have important consequences at the physiological pH of 7.4 (Ross and Riley 1994; Kawai et al. 1996). Whereas the fluoroquinolones norfloxacin, ofloxacin, and ciprofloxacin exist 90% in the zwitterionic and 10% in the anionic form, grepafloxacin exists only 75% in the zwitterionic form, with 20% in the cationic and 5% in the anionic form (Rodrigues et al. 2002), moxifloxacin exists 93% in the zwitterionic and 7% in the cationic form and Erx exists 70% in the zwitterionic form and 30% in the

anionic form. The spectral absorption data obtained for Erx, at pH 7.4, are in agreement with spectra previously described (Saraiva et al. 2010; Lizondo et al. 1997) presenting the characteristic absorption band at 271 nm and two smaller bands at 324 and 336 nm. The observed characteristics of the fluorescence spectrum are $\lambda_{exc} =$ 272 nm and $\lambda_{em} = 413$ nm. These spectral properties of Erx are very similar to those of other fluoroquinolones (Meras et al. 1998; Drakopoulos and Ioannou 1997; Djurdjevic et al. 1995).

The spectra obtained for the metalloantibiotic exhibited a 5-nm bathochromic shift in the maximum wavelength, compared to the spectra of free Erx and Phen, which is also in agreement with previous observations (Saraiva et al. 2010; Efthimiadou et al. 2006). Solution behavior of metalloantibiotic shows the predominance, at pH 7.4, of the species [CuErxPhen] 42% and [CuHErxPhen] 37% (HErx and Erx represents Erx in its zwitterionic and its anionic form, respectively). The observed characteristics of the fluorescence spectrum are similar to those of Erx alone, $\lambda_{exc} = 272$ nm and $\lambda_{em} = 412$ nm.

Size distribution of extruded liposomes was determined and the results are shown in Table 1. Independently of the lipid system understudy the liposomes exhibit a size around 100 nm. The small differences observed are the result of differences in the packing behavior of the highly nonhomogeneous lipidic systems of *E. coli* lipid extracts, which present an increased diversity of the lipids present and also different thermotropic behavior (Lopes et al. 2010).

The emission fluorescence spectra vs. lipid concentration of Erx and its metalloantibiotic (a ternary complex of Erx/Cu(II)/Phen) are depicted in Fig. 1. The results show that for Erx and for its metalloantibiotic a small shift of the λ_{em} to higher wavelengths—bathochromic shift—with increasing lipid concentration is observed, which can be attributed to an effective lipid–antibiotic interaction (Pávez et al. 2007). Shift in the λ_{em} values are normally attributed to different polarities regarding the environment of the fluorophore; a bathochromic shift is normally observed when the fluorophore changes to a more polar environment. Our results show that this shift is more pronounced for both *E. coli* extracts and for the POPE:POPG:CL mimetic system (7 nm and 9 nm shift for Erx and its metalloantibiotic, respectively) than for the binary mimetic system composed of POPE:POPG (3 and 6 nm shift for Erx and its metalloantibiotic, respectively) (Fig. 2). These results correlate well with an aprotic medium of relatively high polarity, as might be expected from a molecule in a shallow position in a liposome bilayer (Weitman et al. 2001), being the polarity higher in presence of CL. For all the lipidic systems studied the Erx FI decreases with increasing lipid concentration but for the metalloantibiotic an increase in the FI is observed, with increasing lipid concentration. This behavior was also detected for some FQ in different polar solvents and in different mixtures of aqueous–organic solvent, being these changes attributed to a different change of the dipole moment of the molecule upon electronic transitions. This is a consequence of specific interactions between solvent and solute that will influence the nonradiative and radiative coupling between ground and excited state (Pávez et al. 2007; Park et al. 2004; Bilski et al. 1998; Cuquerella et al. 2006).



Scheme 1 Enrofloxacin chemical structure

The obtained values for the partition coefficients (K_p), in the different lipidic systems are summarized in Table 2. The K_p values obtained for the metalloantibiotic are higher than the values obtained for Erx for all the systems studied, a result that could be expected as all the systems have negatively charged head groups that can promote an electrostatic interaction with the positively charged species, that exists at physiological pH, of the metalloantibiotic.

For Erx not only the K_p values are smaller but they change slightly for all the systems used. These results can also be explained on the basis of the Erx species that are present at physiological pH; a repulsion (negatively charged 30%) and a moderate interaction (zwitterionic,

Table 1 Size values (nm) and respective polydispersion index determined by DLS of liposomes prepared by the extrusion method of *E. coli* total lipid extract, *E. coli* polar lipid extract, POPE:POPG:CL (0.67:0.23:0.21), and POPE:POPG (0.75:0.25) molar ratio, in 10 mM HEPES buffer, 0.1 M NaCl, pH 7.4 at 37°C

Lipid model system	Medium diameter (nm)	Polydispersion index
POPE:POPG (0.75:0.25)	114.0	0.10
POPE:POPG:CL (0.67:0.23:0.1)	112.7	0.09
E. coli polar lipid extract	123.7	0.10
E. coli total lipid extract	125.4	0.08

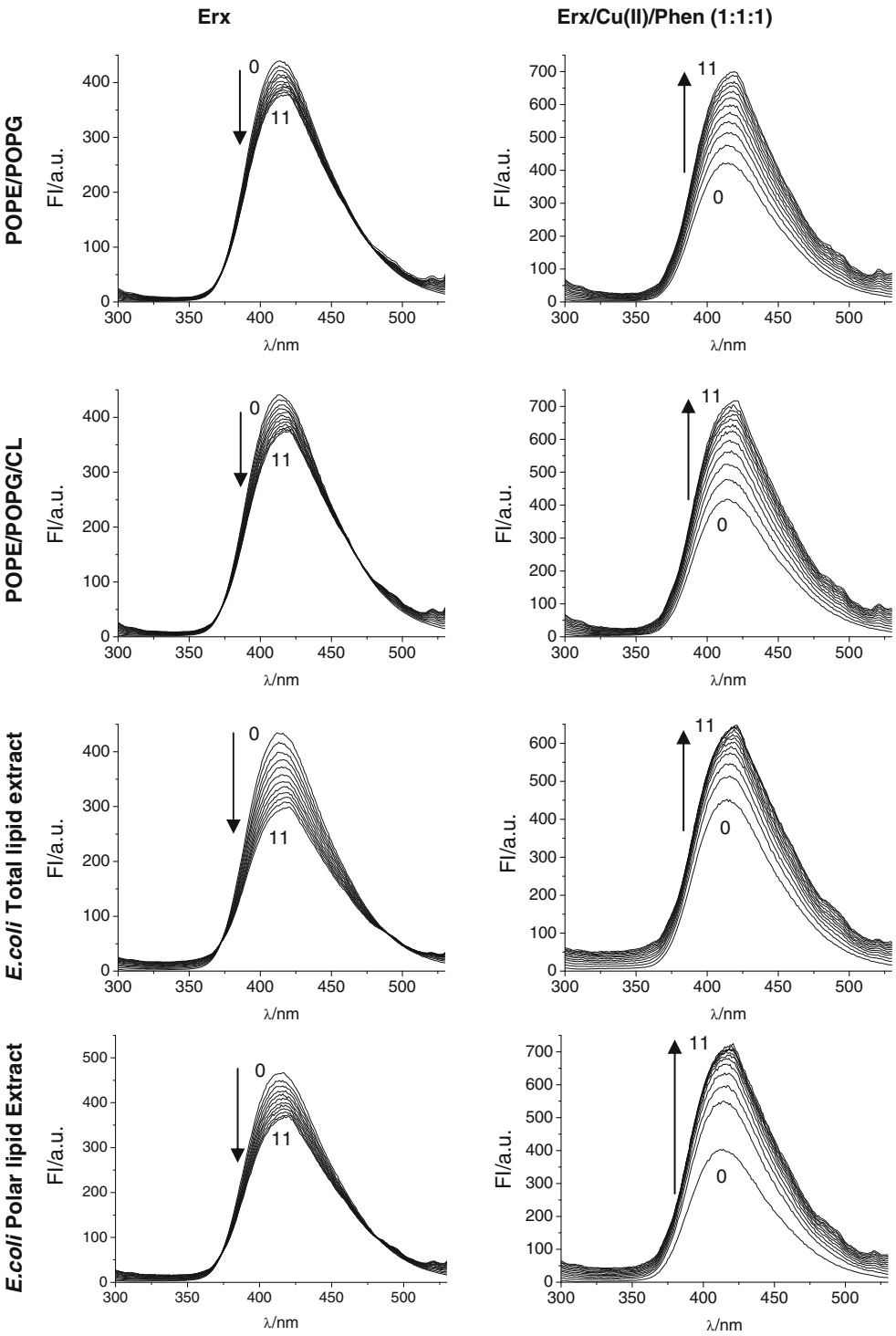


Fig. 1 Emission fluorescence spectra of enrofloxacin (Erx) (8.7 μM) and its ternary complex Erx/Cu(II)/Phen (9.0 μM) (1:1:1) in the absence (0) and presence of increasing lipid concentrations (11) for all the lipidic systems studied

70%) will be expected with the negatively charged head groups of the lipids used.

These results show that the interactions of these two compounds with lipidic systems are different, as expected from their different hydro/lipophilic characteristics. Furthermore microbiological studies performed with these two compounds (Saraiva et al. 2010) clearly show that although they have similar activity against *E. coli* strains, their route of entry seems to be very different.

Considering the different lipidic systems, the K_p values for *E. coli* polar lipid extract are, more or less, two and half times higher than those obtained for *E. coli* total lipid extract. Moreover, the K_p values obtained for the binary system POPE:POPG (0.75:0.25 molar ratio) are different

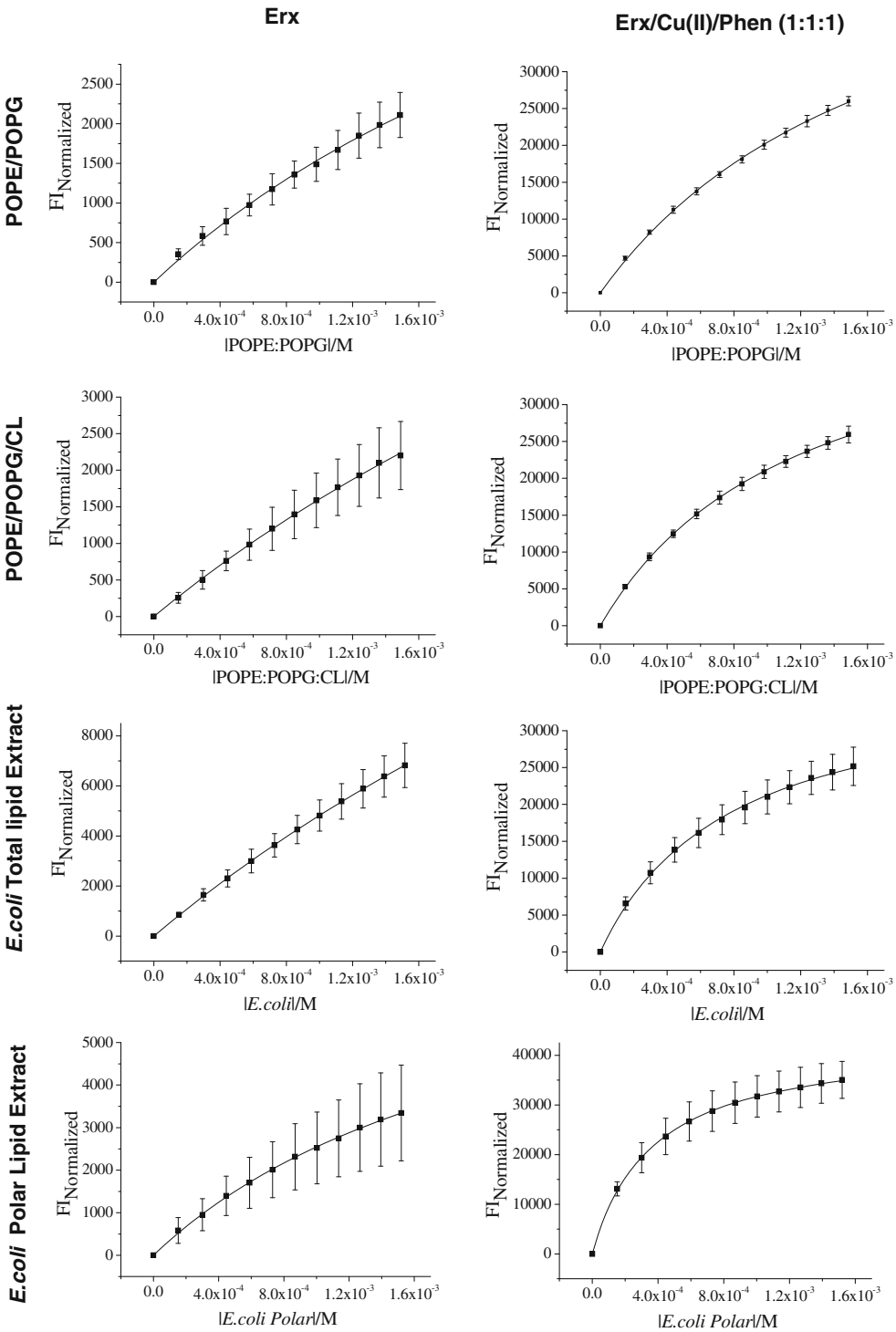


Fig. 2 Partition curves of (Erx) and its metalloantibiotic (a ternary enrofloxacin/copper(II)/phenanthroline (1:1:1) complex) in different LUVs' lipidic systems composed of POPE:POPG (0.75:0.25), POPE:POPG:CL (0.67:0.23:0.10), and polar and total lipid extract of *E. coli*. Equation 3 was fitted to the fluorescence intensity (FI) vs. lipid concentration at a constant antibiotic/metalloantibiotic concentration of approximately 9.0×10^{-6} M. The presented data are the mean of at least three independent measures

from those obtained for the *E. coli* extracts, but for the ternary lipidic system, with POPE:POPG:CL, the K_p values obtained are closer to the ones obtained for *E. coli* total lipid extract, especially for Erx, which is known to interact hydrophobically with *E. coli* membranes (Mahendran et al. 2010). These results are consistent with recent studies (Lopes et al. 2010; Lewis and McElhaney 2009; Haines 2009) where the importance of CL became evident as a relevant constituent since it represents 10% of *E. coli* lipid constitution and with the study that showed that the ternary system composed by POPE:POPG:CL has a thermotropic behavior much closer to the one observed for *E. coli* total and polar extract (Lopes et al. 2010).

The differences in the model systems clearly show that, although, being the binary lipidic system used, in this work, one of the most commonly described model systems in the

Table 2 Partition coefficients of enrofloxacin (Erx) and its metalloantibiotic (a ternary copper(II)/enrofloxacin/phenanthroline (1:1:1) complex) to different LUV systems, determined by fluorescence spectroscopy[a]

Lipid model system	$K_{p(\text{Erx})}$ ($/10^4$)	$K_{p(\text{Cu-Erx-Phe})}$ ($/10^4$)
POPE:POPG (0.75:0.25)	1.50 ± 0.20	3.06 ± 0.12
POPE:POPG:CL (0.67:0.23:0.1)	0.92 ± 0.15	4.68 ± 0.08
E. coli polar lipid extract	2.43 ± 0.13	15.2 ± 023
E. coli total lipid extract	0.88 ± 0.33	7.12 ± 0.28

[a] POPE:POPG:CL (0.67:0.23:0.21) and POPE:POPG (0.75:0.25) refer to the molar lipid ratios. All systems were studied in 10 mM HEPES buffer, pH 7.4, 0.1 M NaCl, at 37°C. The reported values are the mean of at least three independent measures

literature to mimic *E. coli* bacterial membranes (Murzyn et al. 2005; Lohner et al. 2001; Pozo Navas et al. 2005), the addition of CL clearly improves the model, mimicking better the possible interaction that antibiotics can have with *E. coli* membranes but still benefiting from the use of a simpler mimetic system.

Conclusion

To counteract bacterial resistance it is not only important to try to design new antibacterial agents but particularly ones that can have a different route of entrance or a different target. The increased use of Erx at veterinary hospitals and in food animal productions is directly related with the prevalence of its resistance, in what concerns *E. coli* isolates, and the possible use of other antibiotics is of utmost importance. The use of metalloantibiotics (metal complexes FQ derivatives) seems a good route to pursue given they have an antimicrobial effect comparable to that of free FQ but its route of entry seems to be very different. To try to understand this difference it is important to use a good model membrane system of bacterial membranes and to take in consideration lipid thermotropic behavior and membrane surface lipid net charge.

The results obtained in this work show that lipid/antibiotic interactions were sensitive to the phospholipid composition. In all the studied systems the partition coefficients obtained for the metalloantibiotic were always higher than those obtained for Erx. These results indicate that their mechanism of entry should be quite different since an electrostatic interaction between the positively charged species of the metalloantibiotic, which exists at physiological pH, with the negatively charged head groups of the lipids clearly promotes lipid/antibiotic interactions. Moreover, the behavior observed for the mimetic system, PE:PG:CL, is the one that is closer to that observed in *E. coli* total lipid extract pointing out for the fact that the introduction of CL seems to have a significant impact on the surface properties of membranes in which it is incorporated.

As a final conclusion it is important to state that being CL particularly prominent in several Gram-negative bacteria, and given that it is a doubly negatively charged four-tailed phospholipid with a polar headgroup, which is relatively rigid (i.e., it is a mobility-restricted entity) with a relative small cross-sectional area per lipid, its incorporation in model system membranes can have a significant impact on the surface properties of membranes and should be considered when using model system of bacterial membranes.

Acknowledgments Partial financial support for this work was provided by FCT PTDC/SAU-FAR/111414/2009. SCL thanks FCT for a SFRH/BPD/34262/2006 fellowship.

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