ELSEVIER

Biophysical Chemistry 113 (2005) 123–128

Biophysical Chemistry

www.elsevier.com/locate/bpc

Interaction between quinolones antibiotics and bacterial outer membrane porin OmpF

Patrícia Neves[a], Emir Berkane[b], Paula Gameiro[a,*], Mathias Winterhalter[b], Baltazar de Castro[a]

[a]*Requimte, Departamento de Química, Faculdade de Ciências, 4169-007 Porto, Portugal*
[b]*Groupe de biophysique et de biotechnologie des protéines, IPBS/CNRS 205, R: Campo Alegre Route de Narbonne, 31077 Toulouse, France*

Received 19 July 2004; received in revised form 30 July 2004; accepted 6 August 2004
Available online 27 September 2004

Abstract

In these work, we try to establish a relation between the hydrophobicity of some quinolones and their interaction with OmpF. In order to do that, the values of the binding constant of some quinolones of different "generations" with OmpF were determined by UV–visible spectrophotometry and by fluorimetry. Our results show that there is a strong interaction between all the drugs and the protein and that it becomes larger for the last "generation" fluoroquinolones. These results were compared with previous ones obtained for the interaction of these drugs with simpler biomembrane models (liposomes) and it is possible to conclude that some of the quinolones associate preferably with the protein than with these models. This suggests that an interaction drug/porin is, probably, the preferentially used for the latest fluoroquinolones what makes reasonable to believe that a strong affinity for OmpF means a better capacity to transpose the barrier formed by the outer membrane.

Keywords: Binding constants; Fluoroquinolones; Membrane protein; Spectroscopic techniques

1. Introduction

Fluoroquinolone (quinolone) antibiotics are one of the most successful classes of drugs. They are considered to be well tolerated with relatively limited adverse effects so that the use of several compounds of the series in therapy is increasing [1]. This group of antimicrobial agents has a long story of use in clinical practice, first for treating infections caused by gram-negative bacteria (first "generation" quinolones) and more recently new derivatives (second, third and fourth "generation") have been developed with enhanced activity against gram-positive species, including *Streptococcus pneumoniae*, one of the most important pathogens responsible for respiratory tract infections, acute otitis and meningitis [2,3]. Their activity results from the inhibition of homologous type II topoisomerases, DNA gyrase and DNA topoisomerase IV, enzymes that control DNA topology and are vital for chromosome function and replication [2].

The outer membrane of gram-negative bacteria (the protective coat of the bacterial cell) is the major permeability barrier for quinolones to access their target site and to develop their antibacterial activity. Nevertheless, despite their widespread use in clinical therapy, there is a lack of information on the molecular mechanism and kinetics of quinolone entry into the bacterial cell. Several studies with different bacteria strains showed that the outer membrane protein F (OmpF) plays an important role in the uptake of some of these antibiotics, but if the entry of the antibiotic is through the lipid/protein interface, or through the porin channel it is still unknown [4–6]. On the other hand, it must be pointed out that a direct uptake by a lipid mediated pathway, mainly through the phospholipid bilayer, has also been claimed for some of these antimicrobial agents [4,7]. These different pathways appear to be related with fluoroquinolones hydrophobicity; however, the precise

* Corresponding author. Tel.: +351 226082889; fax: +351 226082959.
E-mail address: agsantos@fc.up.pt (P. Gameiro).

0301-4622/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2004.08.004

molecular structure and functional domains of the outer membrane involved in this process remain unknown [4,6,7].

The study of a possible interaction of fluoroquinolones with OmpF protein in different model membranes is important to understand the relationship between the structural properties of the protein and the uptake of these drugs, but moreover to try to relate antibiotic resistance in a quantitative manner to translocation across the outer membrane.

The different affinity of the drugs to lipids and/or protein will be crucial to predict nonlinear pharmacokinetic and stereoselective pharmacokinetic processes, covalent binding to different molecular structures and drug displacement phenomena [8].

Although the interaction of fluoroquinolones with phospholipidic monolayers and liposomes, systems that can mimic well the phospholipid bilayer of biological membranes, are reasonably understood [9–13], there is a lack of information, to the best of our knowledge, about a possible interaction of fluoroquinolones with the outer membrane proteins, porins.

In order to gain some insights on a possible drug/protein association, we studied the interaction of fluoroquinolones with OmpF by selecting representative members of each generation of this larger group of antibiotics and promoting contact with purified OmpF. A possible interaction between the fluoroquinolones and OmpF was studied by spectrophotometry and fluorimetry.

The experimental data confirm the existence of a strong interaction between fluoroquinolone/OmpF and that this interaction grows from the first "generation" fluoroquinolone, nalidixic acid (NA), to moxifloxacin (MX), a fourth "generation" fluoroquinolone. Furthermore, fluorescence spectroscopy shows an interaction between the fluoroquinolones and OmpF tryptophanes. Nevertheless, the reliability of our results come from the different mathematical methodologies applied and could only be achieved by using nonlinear regression analysis at several wavelengths.

2. Materials and methods

Grepafloxacin (GR) was a gift from Glaxo-Wellcom. Ciprofloxacin (CP) and Moxifloxacin were a gift but from Bayer. Nalidixic acid and *N*-(2-hydroxyethyl) piperazine-*N*′ -ethanesulfonic acid (Hepes) were from Sigma. Octylpolyoxyethylene (oPOE) from Bachem and all other chemicals from Merck (*pro analysi*). All solutions were prepared with 10 mM Hepes buffer (0.1 M NaCl, pH 7.4) with 3% of oPOE.

OmpF (outer membrane protein F) was purified from *E. coli*, strain BL21 (DE3) Omp8, following published procedures [14]. OmpF concentration was estimated using the bicinchoninic acid protein assay against bovine serum albumin as standard.

2.1. Spectroscopic measurements

Absorption spectra were recorded with a UNICAM UV-300 spectrophotometer equipped with a constant-temperature cell holder. Spectra were recorded at 37 °C in 1 cm quartz cuvettes with a slit width of 2 nm in the range 230–450 nm. Fluorescence measurements were performed on a Jobin-Yvon FL-3D spectrofluorometer equipped with a CCD-3000 controller and with a constant-temperature cell holder. All the spectra were recorded at 37 °C, with a slit width of excitation and emission of 0.05 mm, an exposure time of 4.000 s in the range from 300 to 550 nm for emission and 280–300 nm for excitation. Same fluorescence measurements were also performed in a Perkin-Elmer spectrofluorimeter also a 37 °C, with a slit width of excitation and emission of 10 nm, in the same wavelengths range.

2.2. Protein–drugs interaction

Sample solutions were prepared by mixing a known volume of protein with a suitable aliquot of drug in Hepes buffer and the corresponding reference solutions were prepared identically, but without protein. All samples were vortexed and incubated at 37 °C for 30 min prior to recording the spectra (UV–visible or the fluorescence). Typically, two sets of 8–12 vials (1.5 ml) were used in each experiment. Drug concentration was in the range 5–35 μM and OmpF concentration ≈2.6 μM for UV–visible. For fluorescence measurements, drug concentration was in the range 2.3–9.54 μM and OmpF concentration 1.5 or 23 μM for OmpF. Studies of a possible interaction of fluoroquinolones with oPOE micelles, in the same experimental conditions, have been performed (results not show) but no interaction could be detected.

2.3. Data analysis

Spectral changes observed from the interaction between the protein and the drugs were first treated by linear and nonlinear graphical methods, using the wavelength of maximum absorption/intensity (using the computer program *Origin* 6.1™) to validate the model and to determine a first approximation of the values of the binding constants. The final values reported were calculated over all the wavelength range, using the programs HYPERQUAD [15] (UV–visible data) and SPECFIT/32™ Global Analysis SystemVersion 3.0 for 32-bit Windows (demo version).

3. Results

There are several general expressions for binding isotherms but it is usually appropriated to test the simplest of these models, namely 1:1 complex formation against the data before adopting more complicated assumptions

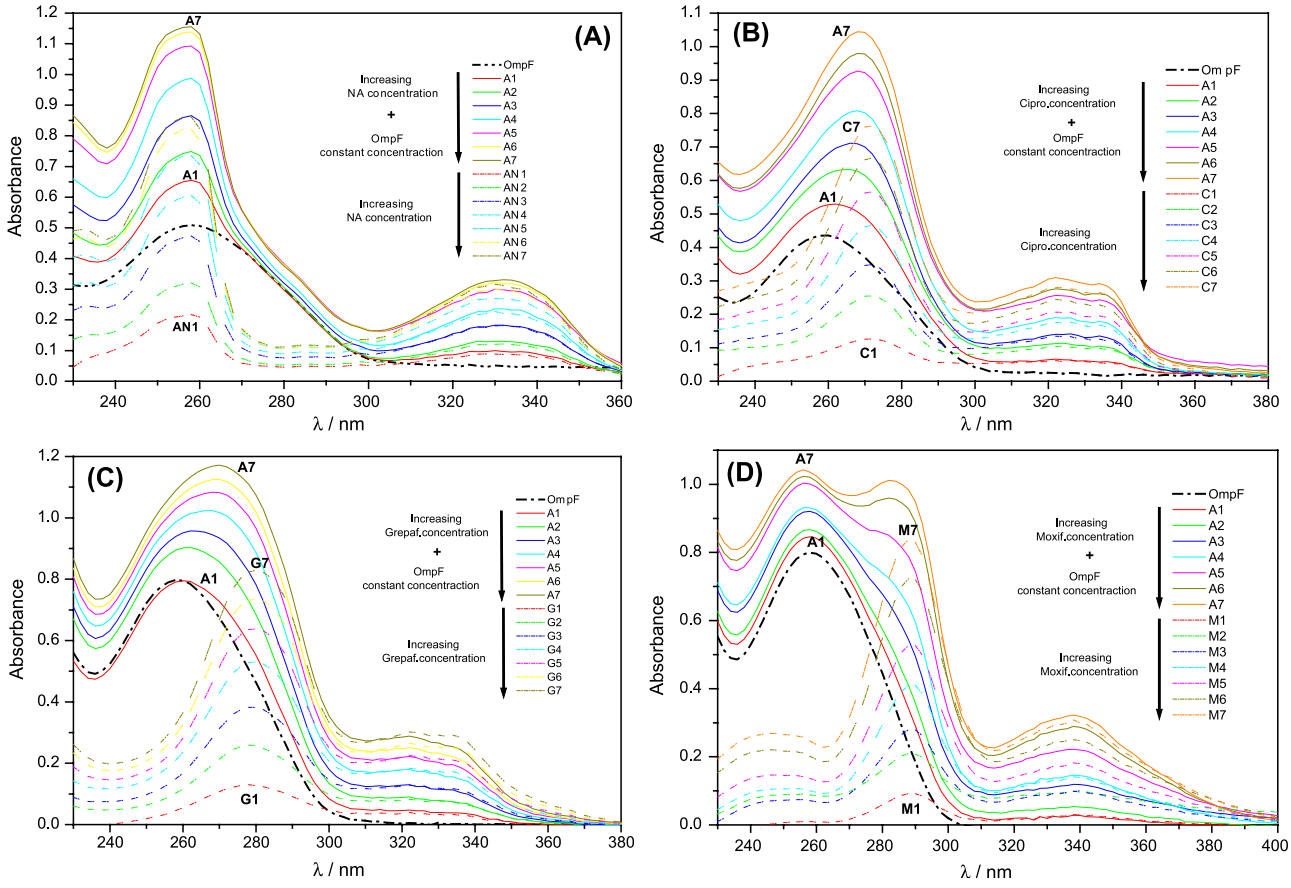

Fig. 1. UV–visible spectra of the quinolones studied in absence (- - -) and presence (—) of OmpF (≈2.6 μM). (A) Nalidixic acid (μM): (1) 12.2, (2) 18.4, (3) 26.2, (4) 36.7, (5) 45.9, (6) 52.0, (7) 55.1; (B) ciprofloxacin (μM): (1) 5.0, (2) 10.0, (3) 14.9, (4) 19.9, (5) 24.9, (6) 29.9, (7) 34.8; (C) grepafloxacin (μM): (1) 5.6, (2) 10.4, (3) 15.2, (4) 21.7, (5) 26.1, (6) 30.4, (7) 34.8; (D) moxifloxacin (μM): (1) 4.8, (2) 7.2, (3) 10.7, (4) 17.9, (5) 20.3, (6) 23.8, (7) 35.8.

[16,17]. In a simple 1:1 binding system, linear and nonlinear treatment have proved to be equally successful and the former provides a definite proof of the applicability of the model. However, these methods rely only on a selected number of wavelengths (typically those of maximum absorption) and a better analysis (and more precise values) can be obtained by using absorbances at several wavelengths and solving simultaneously the set of equations that describe the proposed model to obtain the equilibrium constant that best describes the system.

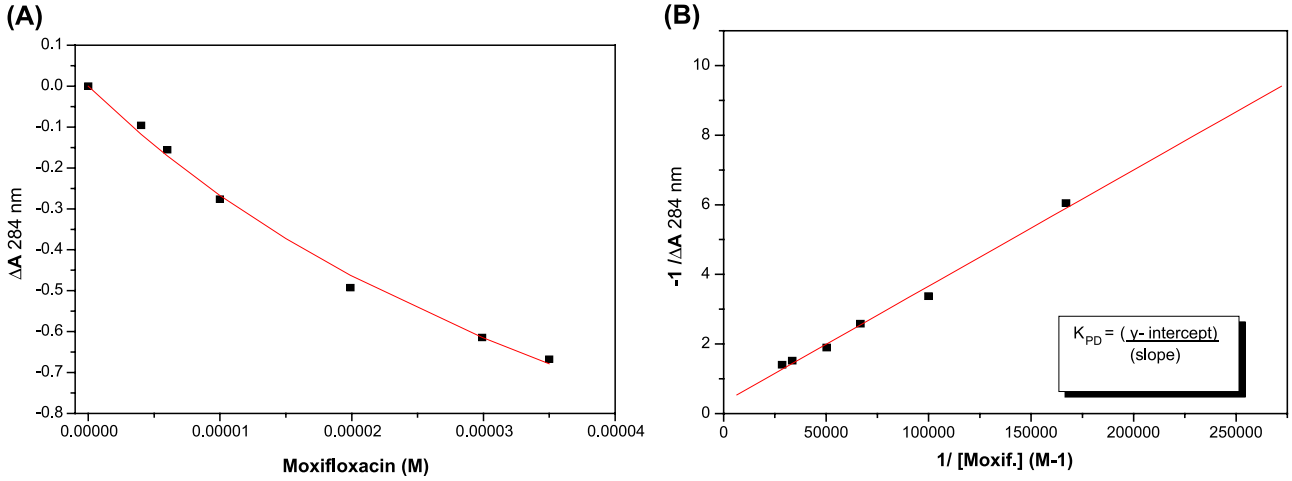

Fig. 2. Graphical treatment of the spectrophotometric data, at λ=284 nm, obtained for the drug Moxifloxacin. (A) nonlinear fit adjusted to Eq. (2) and (B) linear double-reciprocal plot [12].

3.1. Determination of the stability constants by UV–visible data

For the system formed by the quinolone and the protein, where a single 1:1 complex (PD) is formed, the solution absorbance is proportional to solute concentration, $A=\varepsilon_P[P]+\varepsilon_D[D]+\varepsilon_{PD}[PD]$, where [P], [D] and [PD] are the protein, drug and complex molar concentrations, respectively, and ε_P, ε_D and ε_{PD} the corresponding extinction coefficients. The difference between the solution absorbance and that of a solution containing only the protein at the same total concentration, P_t, can be related to the stability constant by the following equation:

$$\Delta A = \frac{P_t \Delta\varepsilon_{PD} K_{PD}[D]}{1 + K_{PD}[D]} \quad (1)$$

where P_t is total concentration of protein and K_{PD} the stability constant of the 1:1 complex.

In Fig. 1 are depicted the spectra for all drugs used in these study in the absence and presence of OmpF and the absorption maxima were used to fit this model (Eq. (1)).

The results obtained for all the drugs show that all data fits well to a 1:1 drug/protein model, and a linear double reciprocal plot and the nonlinear regression plot obtained for one of the drugs are depicted on Fig. 2. For all the other drugs, the plots were similar and the data was obtained for more than 3 independent determinations with 8–12 sample solutions (results available from the authors).

The values of the binding constants obtained by this first approach were then refined by nonlinear least-squares regression with the program *HYPERQUAD* [15], for which it was possible to minimize simultaneously data for several wavelengths. The values obtained for the binding constants are depict in Table 1.

3.2. Determination of the stability constants by fluorescence data

The stability constants were also calculated from fluorimetric data (Fig. 3). OmpF has inherent fluorescence due to the existence of trypthophan residues [18,19], and the fluorescence of these residues is quenched in the presence of drug and this quenching can be attributed to formation of a non-fluorescent complex between the protein and the drug. The fluorescence spectra of all quinolones also exhibit a significant decrease when OmpF is present what is in accordance with the data obtained by UV–visible spectroscopy.

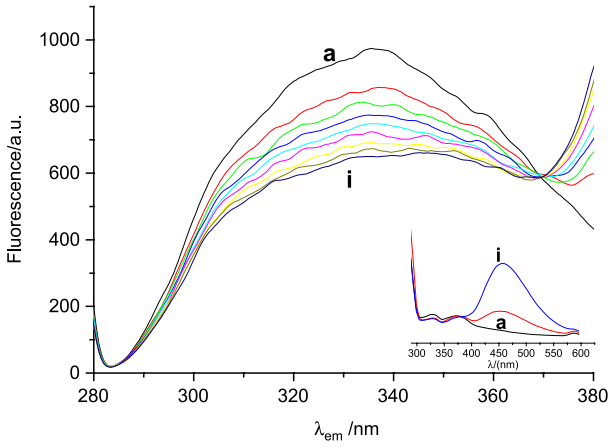

Fig. 3. Fluorescence spectra for increasing concentrations of Ciprofloxacin in absence (a) and in the presence (b–i) of OmpF (1.5 μM). Moxifloxacin concentration (μM): (b) 2.9, (c) 4.2, (d) 5.2, (e) 5.9, (f) 7.0, (g) 8.2, (h) 8.8, (i) 9.9.

The dependence of fluorescence intensity upon drug concentration from static quenching is proportional to the binding constant for complex formation by:

$$\frac{F_0}{F} = 1 + K_{PD}[D] \quad (2)$$

where F_0 and F are the fluorescence intensity of the protein solution in absence and presence of the drug, respectively.

The fitting of this equation to the experimental data yields a straight line, over all concentration range (correlation coefficient always larger than 0.99), implying, as

Table 1
Values of the binding constants obtained for the four drugs with OmpF[a]

Quinolone	log K_{NA}	log K_{CP}	log K_{GR}	log K_{MX}
Generation	1st	2nd	3rd	4th
Hyperquad	4.04±0.02	4.17±0.03	4.24±0.02	4.35±0.03
Graphical[b] methods	3.76±0.39	3.85±0.34	4.02±0.20	4.27±0.25
Graphical[c] methods	3.99±0.15	4.55±0.24	4.66±0.27	4.62±0.35
Fluorescence[d] SPEC	4.02±0.02	4.37±0.05	4.57±0.03	4.65±0.04

[a] The reported values are the mean of at least two independent measurements; the errors are the standard deviation.
[b] Results obtained by Eq. (1).
[c] Results obtained by Eq. (3).
[d] Results obtained with SPECFIT/32™.

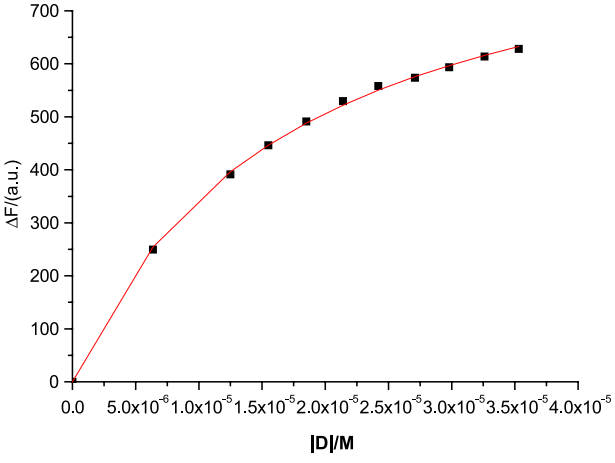

Fig. 4. Graphical treatment of the fluorimetric data, at λ_{exc}=285 nm, obtained for the drug Ciprofloxacin. Nonlinear fit adjusted to Eq. (3).

predicted from the UV–visible data, that the quenching must be static.

Preliminary values for the binding constants were also determined, at the wavelength maxima, by equation

$$\Delta I = \frac{\Delta I_{\max} K_{\mathrm{PD}} |\mathrm{D}|}{1 + K_{\mathrm{PD}} |D|} \quad (3)$$

These results (Fig. 4) were further refined by nonlinear least-squares regression with the program SPECFIT/32™ and the values were identical, within experimental error, to those obtained by UV–visible spectrophotometry. The values obtained are summarized in Table 1.

4. Discussion

The linearity of the double-reciprocal plots (UV–visible) and static quenching plot (fluorimetry) provides a good indication of the reliability of the chosen model. Although the extent to which the experimentally binding curves, and consequently the degree of saturation, for the two spectroscopic methods used were different, the values obtained for the binding constants are identical, within experimental error. The data was treated for all the $\lambda_{\max}$ of the spectra and it is apparent from the values depicted in Table 1 that the binding constants increase from nalidixic acid to moxifloxacin. In principle, a single determination at one wavelength is sufficient to calculate binding constants, especially for a simple system like 1:1 complex formation, but using some data on both sides of the $\lambda_{\max}$ will reduce the effect of noise in the spectrophotometric data, thus providing more precise values for the binding constants. Data from several wavelengths were then analysed with the program Hyperquad and although the values obtained for the binding constants were similar (see Table 1) the precision was always higher.

Independent support for the values of the binding constants determined by UV–visible was provided by the good agreement with the values obtained by fluorimetry. This observation provides additional support to the model used and shows that the values obtained for the binding constant are precise and reliable.

Under the conditions used in this work, OmpF is a trimer, and its fluorescence is due to the presence on its structure of six tryptophan residues (two per monomer at positions 61 and 214) [18–20]. As it is known, fluorescence quenching is often used to gain information about the type of molecular associations surrounding the fluorescent molecule [21–23], so the quenching of the protein fluorescence, besides confirming an interaction with the drug, also suggests that a possible first site of interaction of the drugs, when trying to transpose the hydrophilic channel formed by the trimer, can be the tryptophan residues or the zone surrounding it.

The values obtained for the binding constants of the drugs with OmpF show that there is a strong interaction between all the drugs and the protein and that this interaction increases in the order: nalidixic acid<ciprofloxacin<grepafloxacin<moxifloxacin, which parallels their "generation" (moxifloxacin is fourth "generation"). This result, when associated with the reported reduction of the minimal inhibitory concentration (MIC) for the newer fluoroquinolones [24–27], makes it reasonable to believe that a strong affinity for OmpF means a better capacity to interact with the barrier formed by the outer membrane.

5. Concluding remarks

The results obtained in this work show that all the fluoroquinolones have a good affinity for OmpF and that this affinity is maximal for the last "generation" fluoroquinolones. The analysis of some of the latest studies involving the use of liposomes, as mimetic models for the lipidic side of the biomembranes, shows that the values of the partition coefficients (K_{p}) for some of the more recent fluoroquinolones (like grepafloxacin [9] and moxifloxacin [28]) are very similar. Conjugation of these results with those obtained in this work show that the association of these drugs in presence of OmpF is higher, and different for different drugs, than that found in liposomes, this emphasizes the idea that for the more recent members of this group of drugs the porin hydrophilic pathway can, probably, be the preferred one.

Furthermore, as the strength of the interaction between the fluoroquinolones and the pore forming protein OmpF increases with the drug "generation", which also is associated with a larger spectrum of activity and with smaller MIC values [24–27], it is reasonable to suggest that the enhanced antimicrobial activity of the fluoroquinolones can be related to its ease of access to the transport target (OmpF).

Acknowledgements

Partial financial support for this work was provided by "Fundação para a Ciência e Tecnologia" (FCT, Lisboa) through project POCTI. P.N. thanks FCT for a fellowship (SFRH/ BD/ 6369/ 2001).

References

[1] A. Albini, S. Monti, Photophysics and photochemistry of fluoroquinolones, J. R. Soc. Chem. 32 (2003) 238–250.
[2] E. Pestova, J.J. Millichap, G.A. Noskin, L.R. Peterson, Intracellular targets of moxifloxacin: a comparison with other fluoroquinolones, J. Antimicrob. Chemother. 45 (2000) 583–590.
[3] R. Quintiliani, R.C. Owens, E.M. Grant, Clinical role of fluoroquinolones in patients with respiratory tract infections, Infect. Dis. Clin. Pract. 8 (Suppl. 1) (1999) S28–S41.
[4] J. Chevalier, M. Malléa, J.M. Pagés, Comparative aspects of the diffusion of norfloxacin, cefepime and spermine through the F porin channel of *Enterobacter cloacae*, J. Biochem. 348 (2000) 223–227.

[5] P.G.S. Mortimer, L.J.V. Piddock, The accumulation of the five antibacterial agents in porin-deficient mutants of *Escherichia coli*, J. Antimicrob. Chemother. 32 (1993) 195–213.
[6] D. Hirai, H. Aoyama, T. Irikura, S. Iyobe, S. Mitsuhashi, Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*, Antimicrob. Agents Chemother. 29 (1986) 535–538.
[7] J.S. Chapman, N.H. Georgopapadakou, Routes of quinolone permeation in *Escherichia coli*, Antimicrob. Agents Chemother. 32 (1988) 438–442.
[8] J. Oravcová, B. Böhs, W. Lindner, Drug-protein binding studies. New trends in analytical and experimental methodology, J. Chromatogr., B, Biomed. Sci. Appl. 677 (1996) 1–28.
[9] C. Rodrigues, P. Gameiro, S. Reis, J.L.F.C. Lima, B. Castro, Interation of grepafloxacin with large unilamellar lipossomes: partition and fluorescence studies reveal the importance of charge interactions, Langmuir, ACS 18 (2002) 10231–10236.
[10] S. Merino, J.L. Vázquez, O. Domènech, M. Berlanga, M. Viñas, M.T. Montero, J. Hernándes-Borrell, Fluoroquinolone–biomembrane interaction at the DPPC/PG lipid-bilayer interface, Langmuir 18 (2002) 3288–3292.
[11] M.T. Montero, J. Hernàndes-Borrell, K.M.W. Keough, Fluoroquinolone–biomembrane interactions: monolayer and calorimetric studies, Langmuir 14 (1998) 2451–2454.
[12] A. Grancelli, A. Morros, M.E. Cabañas, Ò. Domènech, S. Merino, J.L. Vazquez, M.T. Montero, M. Viñas, J. Hernández-Borrell, Interaction of 6-fluoroquinolones with dipalmitoylphosphatidylcholine monolayers and liposomes, Langmuir 18 (2002) 9177–9182.
[13] J.L. Vázquez, S. Merino, Ò. Domènech, M. Berlanga, M. Viñas, M.T. Montero, J. Hernández-Borrell, Determination of the partition coefficients of a homologous series of ciprofloxacin: influence of the *N*-4 piperazinyl alkylation on the antimicrobial activity, Int. J. Pharm. 220 (2001) 53–62.
[14] A. Prilipov, P.S. Phale, P.V. Gelder, J.P. Rosenbusch, R. Koebnik, Coupling site-directed mutagenesis with high-level expression: large scale production of mutant porins from *E. coli*, FEMS Microbiol. Lett. 163 (1998) 65–72.
[15] P. Hyperquad Gans, A. Sabatini, A. Vacca, Talanta 43 (1996) 1739–1753.
[16] K.A. Connors, Binding Constants, The Measurement of Molecular Complex Stability, Wileg-interscience, 1987, pp. 147–161.
[17] A.D. Attie, R.T. Raines, Analysis of receptor–ligand interactions, J. Chem. Educ. 72 (1) (1995) 119–124.
[18] B.R. Pattnaik, S.G. Moganty, R. Rajeswari, Selective excitation of tryptophans in OmpF: a fluorescence emission study, Biochem. Mol. Biol. Int. 42 (1997) 173–181.
[19] M.S. Weiss, G.E. Schulz, Porin Conformation in the absence of calcium: refined structure at 2.5 Å resolution, J. Mol. Biol. 231 (Issue 3) (1993) 817–824.
[20] A.H. O'Keeffe, J.M. East, A.G. Lee, Selectivity in lipid binding to the bacterial outer membrane protein OmpF, Biophys. J. 79 (2000) 2066–2074.
[21] A.S. Ladokhin, Evaluation of lipid exposure of tryptophan residues in membrane peptides and proteins, Anal. Biochem. 276 (1999) 65–71.
[22] J.H. Kleinschmidt, L.K. Tamm, Time-resolved distance determination by tryptophan fluorescence quenching: probing intermediates in membrane protein folding, Biochemistry 38 (1999) 4996–5005.
[23] S.M. Raja, S.S. Rawat, A. Chattopadhyay, A.K. Lala, Localization and environment of tryptophans in soluble membrane-bound states of a pore-forming toxin from *Staphylococcus aureus*, Biophys. J. 76 (1999) 1469–1479.
[24] M.E. Jones, M.R. Visser, M. Klootwijk, P. Heisig, J. Verhoef, F.J. Schmitz, Comparative activities of clinafloxacin, grepafloxacin, levofloxacin, moxifloxacin, ofloxacin, sparfloxacin, and trovafloxacin and nonquinolones linozelid, quinupristin–dalfopristin, gentamicin, and vancomycin against clinical isolates of ciprofloxacin-resistant and -susceptible *Staphylococcus aureus* strains, Antimicrob. Agents Chemother. 43 (2) (1999) 421–423.
[25] D. Hardy, D. Amsterdam, L.A. Mandell, C. Rotstein, Comparative in vitro activities of ciprofloxacin, gemifloxacin, grepafloxacin, moxifloxacin, ofloxacin, sparfloxacin, trovafloxacin, and other antimicrobial agents against bloodstream isolates of gram-positive cocci, Antimicrob. Agents Chemother. 44 (3) (2000) 802–805.
[26] J.M. Blondeau, Expanded activity and utility of the new fluoroquinolones: a review, Clin. Ther. 21 (1999) 3–40.
[27] J.M. Blondeau, A review of the comparative in-vitro activities of 12 antimicrobial agents, with a focus on the five new "respiratory quinolones", J. Antimicrob. Chemother. 43 (Suppl. B) (1999) 1–11.
[28] P. Neves, P. Gameiro, B. Castro, "Unpublished results".