Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3871–3889

BIOORGANIC & MEDICINAL CHEMISTRY

Combining Molecular Modeling with Experimental Methodologies: Mechanism of Membrane Permeation and Accumulation of Ofloxacin†

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Received 22 February 2002; accepted 26 July 2002

Abstract—The interaction between ofloxacin, as a model drug of the fluoroquinolone class, and biomembranes was examined as the possible initial step in a transmembrane diffusion process. Dipalmitoylphosphatidylcholine was used for the preparation of biomembrane models. The influence of environmental conditions and protonation on molecular physicochemical behavior, and hence on the membrane interaction, was investigated by differential scanning calorimetry (DSC). This technique has been shown to be very effective in the interpretation of interactions of drug microspeciations with biomembranes. These findings suggest that the interaction occurred owing to ionic and hydrophobic forces showing how the passage through the membrane is mainly favored in the pH interval 6–7.4. It was demonstrated that a pH gradient through model membranes may be responsible for a poorly homogeneous distribution of ofloxacin (or other related fluoroquinolones), which justifies the in vivo accumulation properties of this drug. DSC experiments, which are in agreement with computational data, also showed that the complexing capability of ofloxacin with regard to Mg^{++} or Ca^{++} may govern the drug entrance into bacterial cells before the DNA Girase inhibition and could ensure the formation of hydrophobic and more fluid phospholipid domains on the surface of the model membrane. These regions are more permeable with regard to various solutes, as well as ofloxacin, allowing a so-called 'self-promoted entrance pathway'. The combination of experimental methodologies with computational data allowed a further rationalization of the results and opened new perspectives into the mechanism of action of ofloxacin, namely its interaction with lipid bilayers and drug–divalent cation complex formation, which might be extended to the entire fluoroquinolone class. Ofloxacin accumulation within *Escherichia coli* ATCC 25922 was measured as a function of time. Also in this example, the environmental conditions influenced ofloxacin penetration and accumulation. The in vitro experiments, reported here, show that a suitable balance of hydrophilic and hydrophobic fluoroquinolone properties needs to occur for there to be increased drug permeation.

Introduction

The currently available fluoroquinolones, such as ciprofloxacin and ofloxacin, represent major advances over the original DNA gyrase inhibitor, nalidixic acid.[1] The efficacy of new classes of fluoroquinolones prompted the use of these antibiotics for the treatment and prophylaxis of a variety of bacterial diseases.[2,3] Fluoroquinolones, similar to other clinically useful antibacterial drugs, have intracellular target sites. Therefore, in order to reach their target, these compounds must be able to cross outer and cytoplasmic bacterial membranes. The passage of drugs across the outer membrane of Gram-negative bacteria can occur by diffusion through porin channels. This can take place by facilitated diffusion using specific carriers, and/or by self-promoted uptake.[4,5]

Fluoroquinolones and other classes of antibiotics, which show a certain lipophilicity, are capable of diffusing

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†Preliminary data were presented at the 13th European Symposium on Quantitative Structure–Activity Relationships, Rational Approaches to Drug Design, QSAR 2000 Düsseldorf, Heinrich-Heine Universität, Düsseldorf, Germany, 27 August–1 September 2000.

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PII: S0968-0896(02)00350-4

through lipid bilayers of any biological membrane.[6] The permeability characteristics of these compounds and the active efflux mechanisms in bacterial cells seem to be of particular importance because they can affect the susceptibility of bacterial cells to fluoroquinolones.[7] The transbilayer diffusion of these drugs has been demonstrated,[8] but particular attention should be given to the effect of the charged or protonable/deprotonable groups present in the molecules. In fact, only the uncharged forms of these compounds can diffuse through the lipid bilayers of biological membranes. An initial interaction between the compound and the lipid bilayer is probably the first phenomenon in the diffusion process. This aspect is of particular importance in the penetration of lipophilic agents containing multiple protonation sites into bacterial cells, for example tetracyclines and fluoroquinolones.

Considering that the diffusion of fluoroquinolones through the bilayer structure is not well known, in this paper the interaction of ofloxacin, as a model drug of the fluoroquinolone class, with biological bilayer models was investigated as a function of the environmental conditions, that is pH value, ionic strength and the presence of divalent cations. In fact, it has been shown that a variety of chemotherapeutic agents strongly interact with the phospholipid components of cellular bilayer membranes, demonstrating that drug–membrane interactions seem to have a role in the biological action of several antibacterial and anticancer drugs.[9,10] In particular, the self-promoted 'metal assisted' entrance pathway[5] for fluoroquinolones is based on the removal of divalent cations from the outer membrane. This causes an increase in the permeability of the outer membrane and sensitivity to lysis by the complement.[11] It was hypothesized[12] that fluoroquinolones can interact with the outer membrane as chelating agents. In this case the two vicinal carboxyl–carbonyl groups at C-2 and C-3 of the molecule form a potential divalent-cation-chelating site.[13]

DSC (a powerful tool to investigate interactions of drug microspeciations with biomembranes)[9,14] and molecular modeling methodologies were carried out to gain more insight into the ofloxacin–lipid bilayer interaction and the drug–divalent cation complex formation,[13] which seem to be determinants in the entrance of fluoroquinolone drugs into bacterial cells before DNA gyrase inhibition. This paper presents an analysis of the protonation/deprotonation and chelation behavior of ofloxacin, which could basically be extended to other fluoroquinolone antibacterial agents going through particular aspects which have often been neglected.[13,15] Each of the four possible forms of the ofloxacin (neutral, positively or negatively charged and zwitterionic) was described by the GRID (versions 17, 19 and 20)[16–18] software using water, Ca^{++} and Mg^{++} as probes. The ion probes were selected in order to mimic the chemical nature of the hypothesized ion radius-dependent ofloxacin interaction at the membrane level.[13]

An additional aim of this study was to test how molecular modeling/computational techniques can integrate into and complement experimental techniques such as DSC in investigating dynamic aspects of drug action, e.g., membrane permeation and drug delivery, that is how multidisciplinary approaches can be applied to deal with pharmaceutical technology and drug delivery problems so extending the area of the molecular modeling traditionally linked to the more *static* 3-D QSAR applications.

Results

The presence of two different protonable functional groups in the chemical structure of the ofloxacin molecule can greatly influence the physicochemical properties of this drug and also of all other structurally similar fluoroquinolones.[13,15] In fact, as shown in Figure 1, the environmental pH remarkably influenced ofloxacin solubility and partition. The octanol–water partition experiments showed that the ofloxacin–biological structure interaction may also be influenced and modulated by the pH value. When ofloxacin is fully protonated (pH 6.1: positively charged piperazine N4 and undissociated carboxyl group)[15] or deprotonated, an increase in its hydrophilicity is achieved. As a consequence, a reduction of the log P_{oct} values was observed. On the contrary, the highest log P_{oct} was observed at the pH interval 6–7, where the ofloxacin molecule showed the lowest water solubility due to formation of internally compensated zwitterionic and/or purely uncharged forms.[13,15] The log P_{oct} values are generally recognized as descriptors of hydrophobicity in QSAR studies. The real partition coefficient can be thoroughly understood and taken into account by considering the different charged microspecies that can interact with the biological substrate.[19,20]

Ofloxacin affinity versus membranes, under various environmental conditions, is very important in determining drug passage. Phospholipid vesicles were used as biomembrane models.[21] In particular, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine monohydrate (DPPC) membranes, when submitted to DSC analysis, show an endothermic peak due to the transition from the ordered gel state to the more fluid and less packed liquid crystal phase.[22] DSC scans of DPPC vesicles are also characterized by a less intense pretransition peak triggered by the mesophase transition from the gel state to the ripple phase.[22] The presence of a foreign molecule in the environment of DPPC biomembranes can influence the thermotropic behavior of the vesicle as a function of its own physicochemical properties. The presence of ofloxacin at various molar fractions did not alter the DSC profiles of DPPC biomembranes with regard to the main transition peak (Fig. 2). Only the pretransition peak was progressively influenced as a function of drug concentration. The intensity of this peak (enthalpy value = 0.65 kcal mol^{-1}) was gradually reduced up to an ofloxacin molar fraction of 0.03, when the pretransition peak totally disappeared (Fig. 2). These findings showed that ofloxacin can interact at the level of the phospho-diester groups of DPPC via hydrogen and/or ionic bonds. At higher drug molar fractions a smaller interaction at the level of the bilayer hydrophobic zone was observed, as shown by a minor reduction of main transition ΔH values.

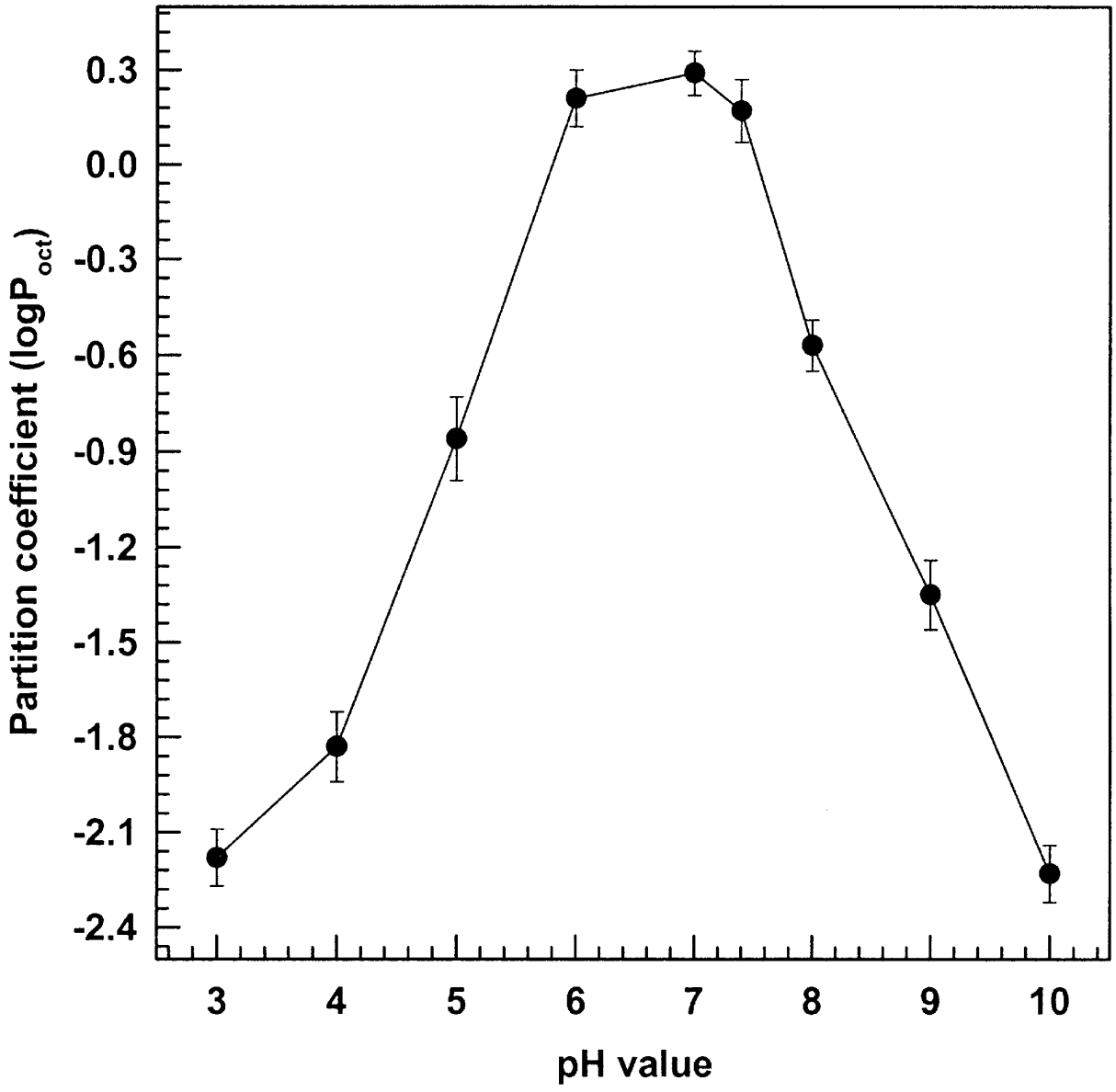

Figure 1. Partition coefficient octanol-water (log P_{oct}) of ofloxacin as a function of pH. The temperature was 37±0.1 °C. Each value represents the mean value of six different experiments ±SD.

The environmental pH can influence the interaction between ofloxacin and DPPC biomembranes. While the solubility studies and the partition coefficient determination demonstrated the overall charge of the ofloxacin molecules (without discriminating between the protonation and deprotonation states of the various functional groups), DSC interaction studies gave a more detailed interpretation of the interaction occurring between ofloxacin and DPPC biomembranes as a function of the drug microspeciations related to pH. Therefore possible interaction pathways were investigated by evaluating thermotropic property variations.

As shown in Figure 3, a broadening of the main transition peak and the disappearance of the pretransition peak were observed at low pH values. The noticeable broadening of the peak was due to a drastic reduction in the co-operativity between DPPC molecules. These findings show a strong interaction of ofloxacin with the phosphocholine headgroups of phospholipid-forming bilayers. The strong co-operativity reduction, mainly at the level of the phospholipid headgroups, also caused a shift of the main peak temperature to lower values, also implying a reduction of enthalpy (ΔH) values (Table 1). Under acidic conditions (below a pH of 6.1) ofloxacin is hydrophilic (Fig. 1), being positively charged on the piperazine N4 with the carboxyl group undissociated.[13,15] The positively charged drug may interact with the negative phosphate moiety of the phosphocholine phospholipid headgroups; these lose the characteristic of zwitterionic molecules and present only a positive charge at the site of the choline group.[13] In this case, the phospholipid headgroups undergo an electrostatic repulsion eliciting both a reduction of the transition energy (ΔH) and a disorganization of the ordered bilayer structure. The drug–bilayer interaction is so strong that a lipid phase segregation was observed in DPPC biomembranes prepared at pH 3.0 (see shoulder

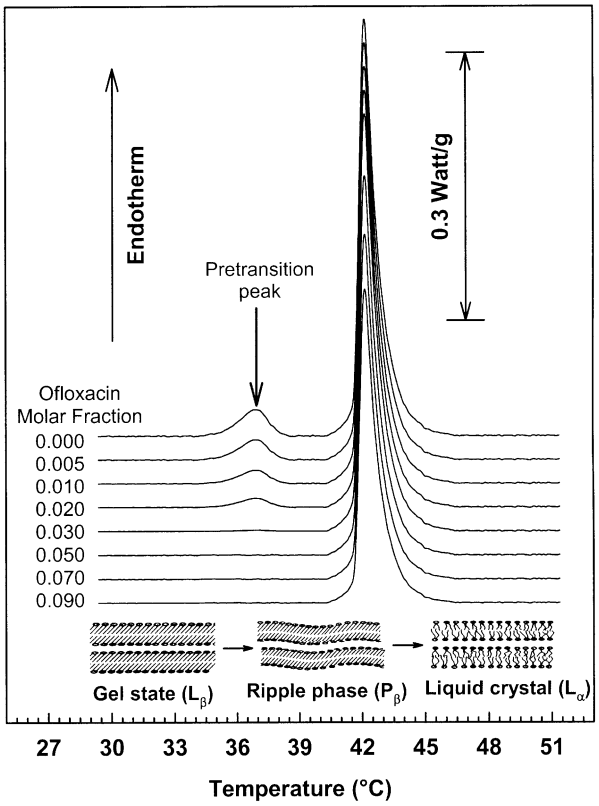

Figure 2. DSC curves of DPPC membrane colloidal dispersions prepared in the presence of different molar fractions of ofloxacin at pH 7.4. The experimental scanning rate was 1 °C/min. The black arrow indicates the pretransition peak. An illustration of the typical phase transition from the gel state to ripple phase and then to the liquid crystal state is also reported at the bottom of the figure.

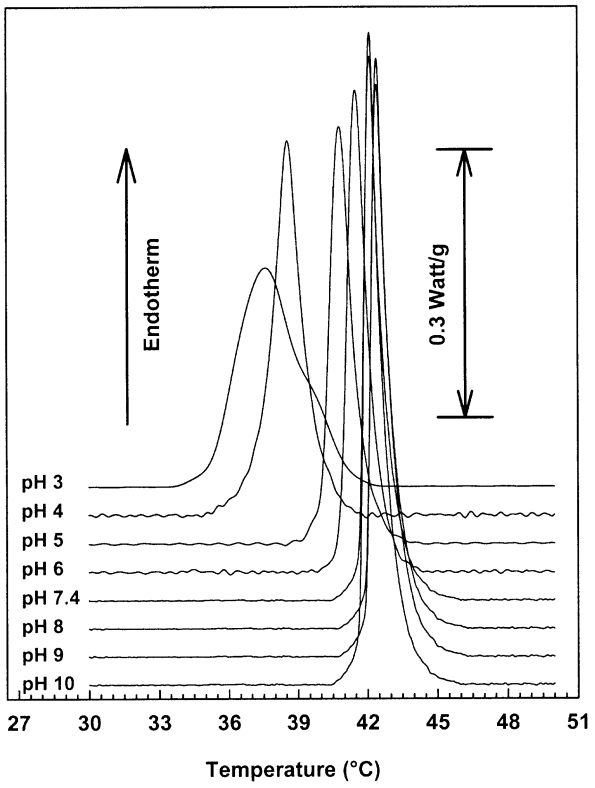

Figure 3. DSC scans in heating mode of DPPC membranes prepared in the presence of ofloxacin (0.07 molar fraction) as a function of the environmental pH value. The scanning rate was 1 °C/min.

of DPPC scans at pH 3.0 in Fig. 3). In fact, the DSC scan of DPPC biomembranes prepared in the presence of ofloxacin at pH 3.0 may be suitably fitted by two different curves, one centered to 37.5 °C and the other to 39.8 °C, highlighting the formation of drug-rich and drug-poor domains, respectively (Fig. 4).

On increasing the pH value, the DPPC peak became sharper with an increase in ΔH of the main transition. The transition peak temperature (T_m) shifted towards 42.1 °C corresponding to the T_m value of pure DPPC. These findings were probably due to a reduction in the population of ofloxacin molecules presenting a positive charge as a function of the pH. Therefore, the drug–DPPC biomembrane electrostatic interactions decrease, indicating a lesser influence of the thermotropic behavior of DPPC vesicles. In the pH values ranging from 6 to 8 no particular variation of T_m and DSC scan shape was observed (Fig. 3, Table 1). In this pH interval, a small ΔH reduction compared with pure DPPC was observed. Under these conditions, the drug is in its higher hydrophobic form and, hence, able to permeate bilayers, slightly perturbing the hydrophobic zone of the membrane. The absence of other kinds of thermotropic variations showed that the interaction was restricted to this level.

Further increasing the pH values towards basic conditions, the drug elicited an increase of ΔH in the pH interval 8–10 (Table 1). In contrast to the behavior under acidic conditions, the shape of DSC traces and main transition temperature were maintained under basic conditions (Fig. 3). In this situation, the negatively charged molecules of ofloxacin can interact with positively charged choline headgroups of DPPC bilayers, acting as counterions, thus eliciting a lowering of the inter-phospholipid electrostatic repulsion forces and, hence, a tight packing of bilayers.

Table 1. Thermotropic parameters of DPPC membranes prepared in the presence of ofloxacin (0.07 molar fraction) with regard to the phase transition from the gel to the liquid crystal state as a function of the medium pH[a]

pH value	T_m (°C)[b]	ΔH (kcal/mol)	Transition range[c]	
			$T_{10\%}$ (°C)	$T_{95\%}$ (°C)
Blank[d]	42.2	−9.34	41.7	43.6
3	36.9	−6.51	35.7	39.9
4	38.5	−7.43	36.4	39.7
5	40.7	−8.21	40.1	42.4
6	41.4	−8.76	41.5	43.9
7.4	42.1	−9.22	41.8	43.7
8	42.3	−9.19	41.7	43.8
9	42.3	−9.79	41.6	43.6
10	42.1	−10.12	41.2	43.7

[a]Data come from the second DSC scan in heating mode. Each value is the average of three different experiments.
[b]T_m, main transition peak temperature.
[c]Transition range temperatures, when 10 and 95% of the sample underwent phase transition.
[d]DPPC vesicles prepared in the absence of ofloxacin in pH 7.4 phosphate buffer. No variation of the thermotropic properties were observed in the pH interval 3–10.

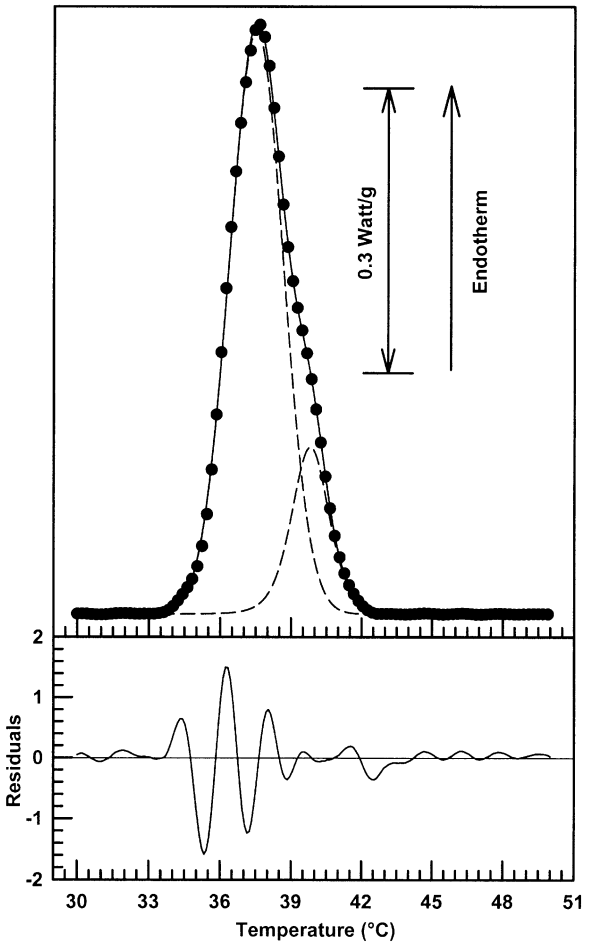

Figure 4. The experimental DSC curves of DPPC membranes prepared in the presence of ofloxacin (0.07 molar fraction) at pH 3 were suitably theoretically fitted. The deconvolution showed the presence of two peaks due to ofloxacin–DPPC phase segregation into drug-rich (peak at 37.5 °C) and drug-poor (peak at 39.8 °C) domains. Keys: •, experimental points; ———, theoretical fitting; – – – –, deconvolved peaks. The fitting procedure is characterized by an r^2 coefficient of 0.99967. At the bottom of the figure the residuals of the fitting procedure are shown.

The interaction of ofloxacin at pH 7.4 with negatively charged membranes made up of DPPC–DPPA (1,2-dipalmitoyl-sn-glycero-phosphatidic acid sodium salt) (8:2 molar ratio) was also investigated. As shown in Table 2, the presence of ofloxacin caused a shift of the main transition peak temperature towards higher values as a function of drug concentration. Ofloxacin also caused an increase of the ΔH values. These findings show that a drug interaction was occurring at the level of the phospholipid headgroups. The interaction probably occurs between the basic amino group of the piperazine ring of ofloxacin and the acid phosphate group of DPPA. This leads to a reduction of the inter DPPA repulsion forces and hence to a tighter bilayer packing. On increasing the drug molar fraction, a broadening of the transition peak was observed (loss of co-operativity; see transition range values, Table 2), probably as a consequence of the phospholipid phase segregation in the DPPC–DPPA–ofloxacin-rich domain and the DPPC–DPPA–ofloxacin-poor domains. This hypothesis is supported by a DSC peak deconvolution

Table 2. Thermotropic parameters of DPPC–DPPA (8:2 molar ratio) membranes prepared in the presence of different molar fractions of ofloxacin with regard to the phase transition from the gel to the liquid crystal state[a]

Molar fraction	T_m (°C)[b]	ΔH (kcal/mol)	Transition range[c]	
			$T_{10\%}$ (°C)	$T_{95\%}$ (°C)
0.00	43.5	−10.61	42.5	44.6
0.01	43.8	−10.92	42.5	44.9
0.02	44.1	−11.27	42.7	45.6
0.03	44.5	−11.61	43.9	47.2
0.05	45.4	−14.88	44.1	48.6
0.07	46.3	−16.61	44.4	49.6

[a]Data come from the second DSC scan in heating mode. Each value is the average of three different experiments.
[b]T_m, main transition peak temperature.
[c]Transition range temperatures, when 10 and 95% of the sample underwent phase transition.

(Fig. 5), which showed the formation of two distinct peaks, one centered at 46.3 °C and the other centered at 44.4 °C.

The release of ofloxacin from unilamellar membranes under different pH conditions was investigated to evaluate in vitro the ability of the drug to pass through biological membranes as a function of protonation/deprotonation states. For these experiments, ofloxacin was solubilized in different pH buffers and encapsulated within model biological membranes. The release medium was always a pH 7.4 phosphate buffer. As shown in Figure 6, different drug protonation states led to different release profiles. Namely, the fastest release rate was observed at pH 7.4 (when the drug is in its hydrophobic form), whereas the lowest release rate was at pH 5. This finding is in agreement with DSC data and log P_{oct} experiments. A certain role in drug permeability was also played by the phospholipid component. In fact, ofloxacin release from 1,2-dimyristoyl-sn-glycero-3-phosphocholine monohydrate (DMPC) membranes was faster than from DPPC membranes (Fig. 6).

To evaluate the capability of ofloxacin to chelate divalent cations, fluorescence spectra at different Mg^{++} and Ca^{++} concentrations were recorded. As shown in Fig. 7, the presence of Mg^{++} or Ca^{++} resulted in an increase in ofloxacin fluorescence at λ_{em} 454 nm compared with that of the control. Ofloxacin fluorescence was altered with Mg^{++}/drug molar ratios as low as 0.05. The presence of monovalent cations, such as K^+ and Na^+, elicited no fluorescence change even when they were present at high concentrations. In the case of Mg^{++} and Ca^{++}, the ofloxacin fluorescence changes were concentration dependent and characterized by saturation at high ionic concentrations (Fig. 7).

Changes in the fluorescence of chelating agents are often related to the binding of metals and have been used to measure levels of cations in biological systems.[23] In the case of ofloxacin, the fluorescence changes observed in the presence of both Mg^{++} and Ca^{++} provided evidence of the formation of complexes with these two cations.

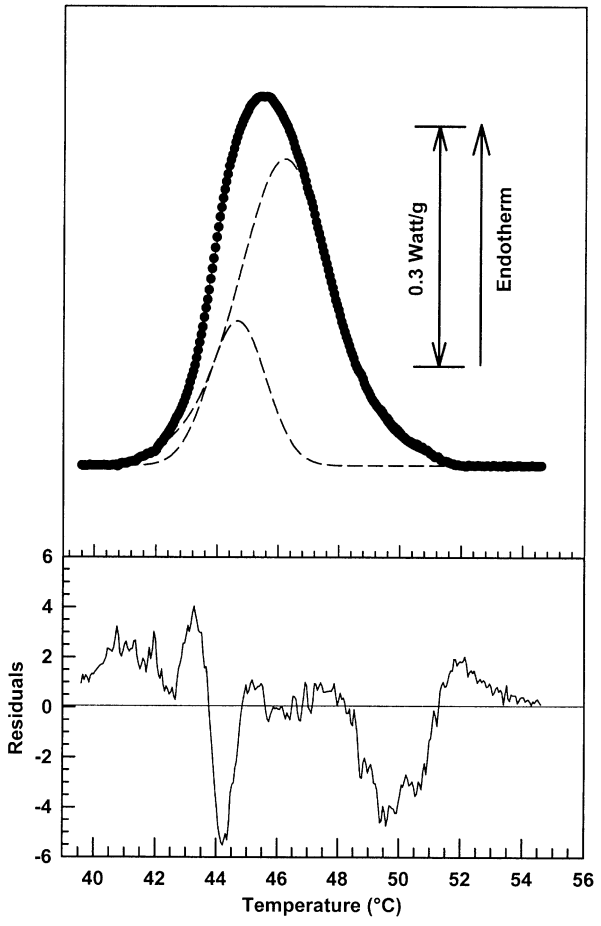

Figure 5. The experimental DSC curves of DPPC–DPPA (8:2 molar ratio) membranes prepared in the presence of 0.05 molar fraction ofloxacin at pH 7.4 were suitably theoretically fitted. The deconvolution showed the presence of two peaks due to the formation of DPPC/DPPA–ofloxacin-poor (peak at 44.4 °C) and DPPC/DPPA–ofloxacin-rich (peak at 46.3 °C) domains. Keys: •, experimental DSC points; ———, theoretical fitting; – – – –, deconvolution peaks. The fitting procedure is characterized by an r^2 coefficient of 0.99934.

The effect of divalent cations (Mg^{++} and Ca^{++}) and their ofloxacin complexes on DPPC membranes was also evaluated by DSC analysis. In these experiments, DPPC membranes were prepared in the presence of Ca^{++} at a concentration of 24 mg/L, which corresponds to the extracellular calcium concentration (very low calcium levels are present within cells). In the case of Mg^{++}, two different concentrations were used, that is, 4.2 and 348 mg/L, which correspond to extracellular and intracellular magnesium concentrations, respectively. As reported in Table 3, the presence of both Mg^{++} and Ca^{++} elicited a shift in the main transition temperature of DPPC membranes to higher values and an increase in ΔH values. These findings may be due to an interaction of the polar phospholipid headgroups with these two cations, leading to a stabilization of the bilayer structure; a more compact phospholipid packing was achieved. These two cations may behave as phospholipid phosphate counter ions. When ofloxacin was present at a molar fraction of 0.07, membranes prepared in the presence of both Mg^{++} (7.2 mg/L) and Ca^{++} showed thermotropic properties close to pure DPPC membranes. In fact, both transition temperature

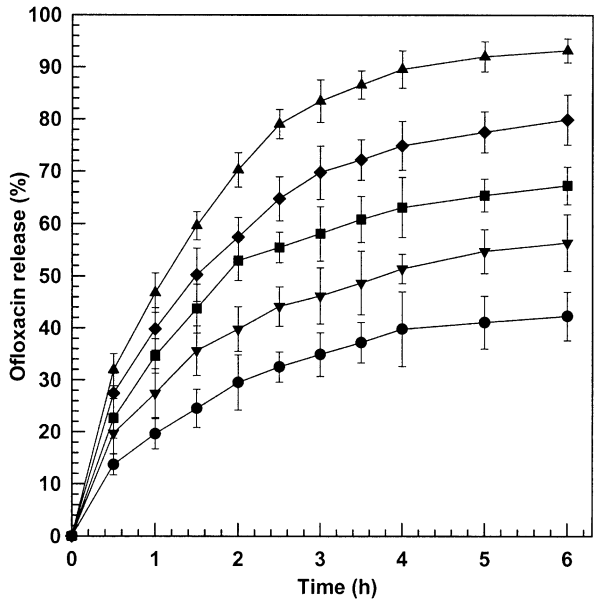

Figure 6. Ofloxacin permeability through membranes suspended in pH 7.4 phosphate buffer as a function of protonation/deprotonation drug situation (internal membrane environment at different values of pH) or phospholipid component. The release experiments were carried out at 37±0.1 °C. Ofloxacin-loaded DPPC membranes at different values of pH: ◆, pH 7.4; ■, pH 6; ▼, pH 5; ●, pH 4. Ofloxacin-loaded DMPC membranes at pH 7.4, ▲. Each point is the average of six experiments±standard deviation.

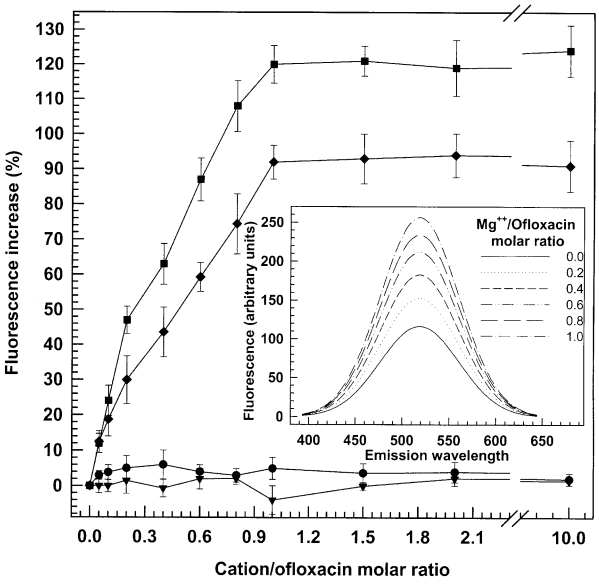

Figure 7. Percentage of ofloxacin fluorescence increase determined as spectra AUC, as a function of the presence of divalent or monovalent cations at different concentrations. The inner plot shows fluorescence spectra of ofloxacin in the presence of Mg^{++} at different molar concentrations. The fluorescence spactra were recorded at 37 °C. Each point represents the average of five different experiments±SD. Keys: •, Na^{+}; ▾, K^{+}; ◆, Ca^{++}; ■, Mg^{++}.

and ΔH moved to control values (Table 3). These findings may be due to the formation of Mg^{++}– and Ca^{++}–ofloxacin complexes, which abstract these cations from the phospholipid polar heads.

The effect of the presence of ofloxacin on thermotropic properties was negligible, when DPPC membranes were

Table 3. Thermotropic parameters of DPPC membranes suspended in saline solutions containing divalent cations (Mg^{++} and Ca^{++}) at different concentrations both in the absence and in the presence of ofloxacin at a 0.07 molar fraction with regard to the phase transition from the gel to the liquid crystal state[a]

Sample	T_m (°C)[b]	ΔH (*K*cal/mole)	Transition range[c]	
			$T_{10\%}$ (°C)	$T_{95\%}$ (°C)
Blank[d]	42.2	−9.34	41.7	43.6
Ca^{++} (24 mg/L)	43.1	−10.67	42.6	44.9
Ca^{++} (24 mg/L) + drug	42.3	−9.42	41.6	43.6
Mg^{++} (7.2 mg/L)	43.0	−10.49	42.3	44.7
Mg^{++} (7.2 mg/L) + drug	42.1	−9.29	41.8	43.7
Mg^{++} (348 mg/L)	43.3	−10.71	42.4	45.4
Mg^{++} (348 mg/L) + drug	43.4	−10.73	42.5	45.2

[a]Data come from the second DSC scan in heating mode. Each value is the average of three different experiments.
[b]T_m, main transition peak temperature.
[c]Transition range temperatures, when 10 and 95% of the sample underwent phase transition.
[d]DPPC vesicles prepared in the absence of ofloxacin and divalent cations in isotonic saline solution.

prepared in the presence of Mg^{++} at a concentration of 348 mg/L (intracellular concentration). Under these conditions, the amount of ofloxacin was not enough to make a complex with all the Mg^{++} cations. In fact, a stoichiometric ratio of 1:1 was proposed for divalent cation–fluoroquinolone complexes.[24]

To confirm the influence of ofloxacin on membrane permeability in the presence of Mg^{++}, the leakage of calcein (a fluorescent probe) from DPPC vesicles was evaluated at Mg^{++} concentrations of both 7.2 and 348 mg/L. As shown in Figure 8, the release of calcein was greater and faster in the presence of ofloxacin at a Mg^{++} concentration of 7.2 mg/L; whereas, at a higher Mg^{++} concentration (348 mg/L) the presence of ofloxacin did not influence the release profile of the fluorescent probe.

Further evidence of the existence of a self-promoted route was gained from DSC experiments on mixed vesicles made up of DPPC–DPPA (8:2 molar ratio). A negatively charged phospholipid was used, due to its ability to interact with divalent cations and to the fact that negatively charged phospholipids are normally present in prokaryotic and eukaryotic cells. The presence of both Mg^{++} and Ca^{++} resulted in a shift of the DPPC–DPPA transition peak temperature towards higher values, an increase of ΔH transition values and a reduction of system co-operativity, as shown by transition range values (Table 4). The effects of these two cations on the thermotropic properties of DPPC–DPPA systems were much more pronounced than those on DPPC vesicles, showing their greater interaction with the negatively charged component of the bilayer. Also in this case, the presence of ofloxacin, due to its complexing activity shifted thermotropic values of DPPC–DPPA membrane transition towards control values. The presence of the drug showed no effect at a Mg^{++} concentration of 348 mg/L.

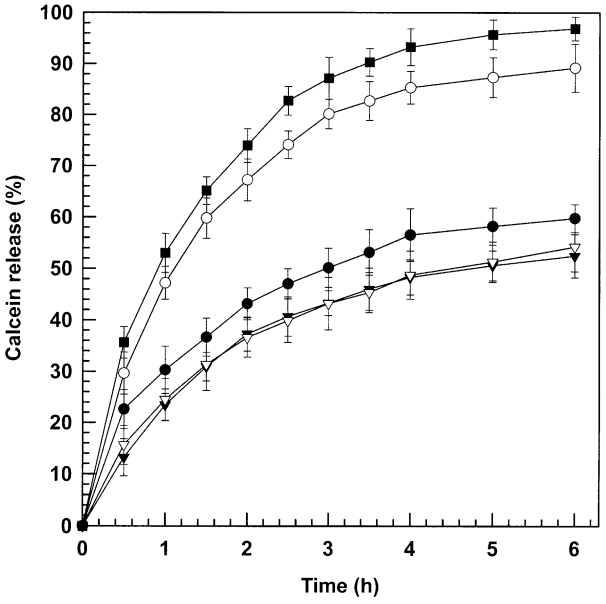

Figure 8. Fluorescent probe calcein release from DPPC membranes both in the presence (hollow symbols) and in the absence (filled symbols) of ofloxacin. Calcein-loaded DPPC membranes were suspended in isotonic saline solution containing Mg^{++} at different concentrations: ○, ● 7.2 mg/L; △, ▲ 348 mg/L. ■, calcein release in isotonic saline solution in the absence of both ofloxacin and Mg^{++}. The fluorescent probe release was carried out at 37 ± 0.1 °C. Each point is the mean value (five experiments) ± standard deviation.

Table 4. Thermotropic parameters of DPPC–DPPA (8:2 molar ratio) membranes suspended in saline solutions containing divalent cations (Mg^{++} and Ca^{++}) at different concentrations both in the presence and in the absence of ofloxacin at a 0.07 molar fraction with regard to the phase transition from the gel to the liquid crystal state[a]

Sample	T_m (°C)[b]	ΔH (kcal/mol)	Transition range[c]	
			$T_{10\%}$ (°C)	$T_{95\%}$ (°C)
Blank[d]	43.5	−10.61	42.5	44.6
Ca^{++} (24 mg/L)	46.8	−12.58	44.1	51.7
Ca^{++} (24 mg/L) + drug	43.9	−10.73	42.7	45.4
Mg^{++} (7.2 mg/L)	48.1	−11.96	44.3	52.0
Mg^{++} (7.2 mg/L) + drug	43.6	−10.49	42.4	45.1
Mg^{++} (348 mg/L)	47.5	−12.04	43.8	52.1
Mg^{++} (348 mg/L) + drug	47.1	−11.87	43.7	51.9

[a]Data come from the second DSC scan in heating mode. Each value is the average of three different experiments.
[b]T_m, main transition peak temperature.
[c]Transition range temperatures, when 10 and 95% of the sample underwent phase transition.
[d]DPPC–DPPA (8:2 molar ratio) vesicles prepared in the absence of ofloxacin and divalent cations in isotonic saline solution.

The initial step in the accumulation of fluoroquinolone antimicrobial agents, that is, binding to cell surface components, is reduced by lowered pH and, under some conditions, by divalent cations.[25] In this study, the accumulation of ofloxacin at a fixed concentration by *Escherichia coli* (ATCC 25922) was examined. A time course of ofloxacin uptake in the absence and in the presence of Mg^{++} ions is shown in Figure 9. Ofloxacin accumulation within *E. coli* in both cases is characterized by a rapid phase of drug entrance followed by a moderate increase. The presence of Mg^{++} ions was responsible

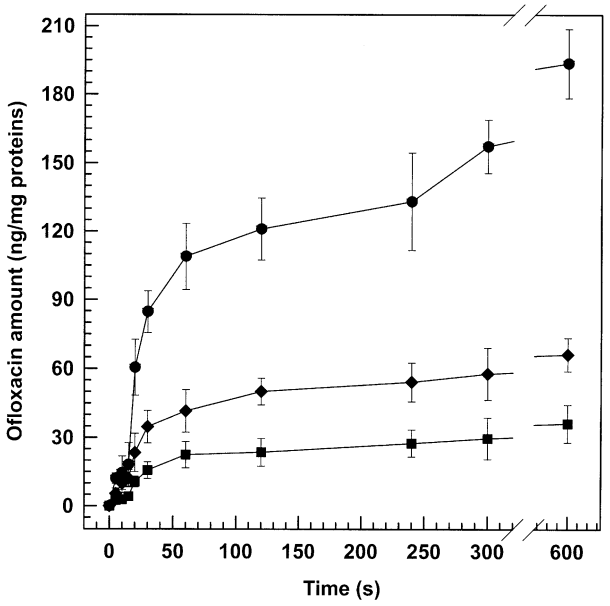

Figure 9. Ofloxacin accumulation within *E. coli* (ATCC 25922) as a function of time either in the absence (●) or in the presence (■) of Mg^{++} ions (20 mM $MgCl_2$) in isotonic phosphate buffer (pH 7.0), or in isotonic acetate buffer (pH 5.5; ◆). Each point represents the mean value of five different experiments ± SD.

for a remarkable reduction of drug uptake (~80%). These findings confirmed that an important role in ofloxacin accumulation is exerted by its divalent cation complexing capability, which may influence, as shown by DSC experiments, bacterial membrane permeability.

The Ofloxacin structure as calculated by an optimization procedure employing molecular mechanics, semiempirical and ab initio methods as implemented in the Spartan[26] and Macromodel[27] software packages was in agreement (Fig. 10) with that in the Cambridge Structural Database (CSD).[28–30] The capability of the applied optimization procedure to detect in silico the intramolecular hydrogen bond that the carboxyl makes to the carbonyl staying in the plane of the ring, as clearly displayed in the CSD structure must be noted (Fig. 10).[28–30] The ofloxacin structure presents a rather flat tricyclic system with a carboxyl group attached to one of the rings, and a carbonyl-type oxygen in an *ortho* position to it. The fact that the ofloxacin carboxyl group can be twisted out of the plane as in the zwitterionic form, because of repulsion with the lone pair of the carbonyl group, might alter the pK_a value. The situation might be further complicated because of the possible additional effect on pK_a due to the heterocyclic ring strain and its orientation, when the molecule approaches the biological target. The zwitterionic form can be assumed as an ensemble of the two possible (positive/negative) charged forms and that *bioactive*, that is with the highest likelihood to exist in physiological conditions.[13,15]

Internally compensated zwitterions, with the proton presumably residing between the carboxylate and phenoxide groups and with a positive charge primarily centered at the piperazine N4, can be present at a physiological pH (Fig. 11). This assignment might make the amine more basic in lipophilic environments. Presumably the quinolone portion, when in the lipid phase, will be more basic in the presence of a divalent cation than a monovalent one.[31]

Some differences in the position of the freely rotating piperazine ring (Fig. 10) between the CSD structure[28–30] and that generated in silico do not affect the overall GRID[16–18] maps. In general, the conformation does not affect the GRID performance when the structure is not very conformationally free as also proved by the similarity between the maps obtained leaving the flexibility option 'on' when compared with 'off' maps (see the Experimental for details on this GRID feature). Calculated rms values between the two *minima* are good and approach a *theoretical* zero value when the piperazine ring is missing from the atom fit procedure as implemented in the SYBYL software.[32] The in silico lowest energy conformer 'free to move' easily docks and adapts to the CSD structure[26–28] as a fixed template using the FLO/QXP module,[33,34] a recently developed molecular design program. A 'point to point' fit with a low strain energy was obtained after few cycles. However, having a crystal structure has long been depicted as the ideal scenario to deal with drug design and generally pharmaceutical problems, though evidence has shown this is not always true and ligand bound forms can differ from those found in the crystal structure, that is bioactives are not always minimum energy conformations.[35]

The calculated heat of formation (ΔH) values of Ca^{++} and Mg^{++} complexes with ofloxacin are numerically meaningless when compared to those generally reported and experimentally validated on a higher stability of the fluoroquinolone magnesium complexes versus calcium ($\Delta H_{Ca} = -223.87$ kcal/mol; $\Delta H_{Mg} = -160.19$ kcal/mol),[13,15] above all considering that the unfavorable effect of the larger calcium cation should be stressed in a gas phase calculation. *Bugs* inherent to the algorithm used in the Spartan suite[26] may lead to the incorrect values. This aspect will be further investigated by computational tests and experimental measurements. However, the positive ΔH values, that is the highest instability of both calcium and magnesium complexes, when acidic conditions are simulated, is conceptually interesting to show the breaking off at a lower pH of the cation complexes hence supporting the proposed entrance mechanism.

When the drug is in a protonated form (positively charged ofloxacin–Ca^{++} or –Mg^{++} complexes were simulated, with the charge residing on the piperazine ring) the formation of complexes is hampered (Fig. 12). In fact, in this situation the ΔH of formation is +83.64 and +162.94 kcal/mol for the Ca^{++} and Mg^{++} complexes, respectively. It may suggest that under acidic pH conditions, such as in the lisosomal compartment and/or in other cellular and *subcellular* compartments, the ofloxacin–divalent cation complex breaks off thus providing the free drug responsible for the antimicrobial action. The *zero approaching* calcium concentration inside the cell *shifts rights* the equilibrium making the complex dissociation easier.

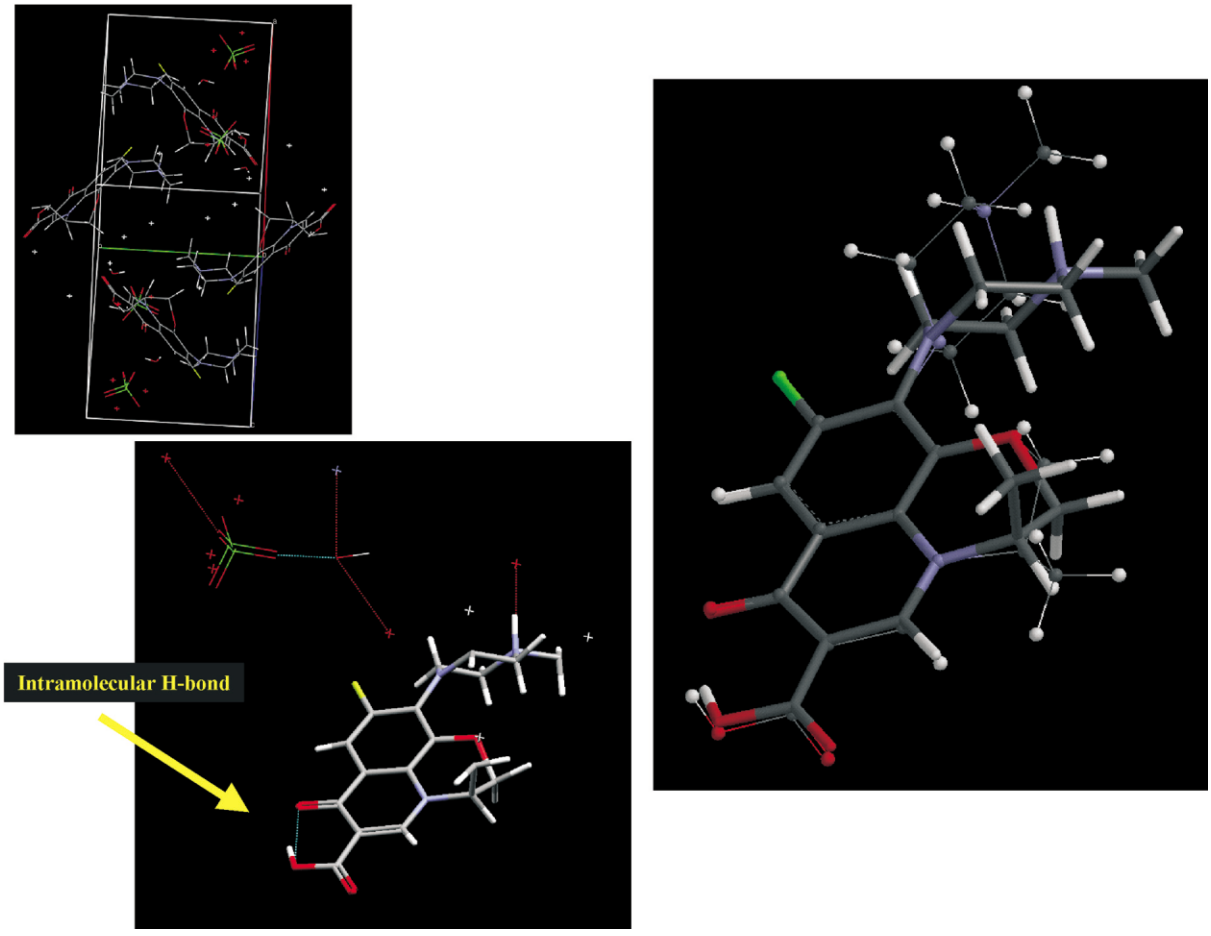

Figure 10. Left side(upper): Ofloxacin (ClO_4^-) crystal packing as in the Cambridge Structural Database (CSD code: SOYBEN).[28–30] Left side(bottom): Ofloxacin crystal structure as in the Cambridge Structural Database (CSD code: SOYBEN).[28–30] The perchlorate anion is also visualized. The latter species was deleted in the pdb file before the GRID analysis (see the Experimental). The intramolecular hydrogen bond is clearly shown (yellow arrow) by CONQUEST.[29,30] The in silico build and optimized structure is clearly consistent with the intramolecular hydrogen bond (see the Results and the Experimental for the explanation). Right side: superposition of the Ofloxacin crystal structure (wire representation) as in the Cambridge Structural Database file (CSD code: SOYBEN)[28–30] with the in silico structure (tube representation). The calculated rms value (atom fit procedure as in SYBYL 6.8)[32] is of 0.502. A quasi theoretical rms value (i.e., approaching 0.0) was obtained missing the piperazine atoms from the fit (see the Results section for the explanation).

Assuming an unprotonated form, that is, not protonated on the piperazine N4, which is assumed as protonated at a physiological pH, although this is not always the real situation or at least it is only partial by which the so called *ambiguous* character of the piperazine ring,[35] an additional heteroatomic site for cation (calcium or magnesium) complex formation was detected by GRID[16–18] in a poorly solvated area as shown by the green contour under the *heterocyclic* (pyrimidine, morpholine and piperazine) region (Fig. 13a,b). The presence of this *unsolvated* area (Fig. 13b), in which the ofloxacin molecule is not exposed to the surrounding 'water', leads one to think that calcium or magnesium cation may form ion radius-dependent complexes by interacting with one of the two possible chromophores (i.e., N,F or O,F) by a kind of *Tarzan-jumping* mechanism between N and O. The involvement of fluorine stems out of the well known preference by hard cations like calcium and magnesium for hard donors.[36] Both fluorine and oxygen show high density at the HOMO orbital (Fig. 14), however the density of the former might be affected because of the phenyl withdrawal effect. In any case, a highly favored five-membered ring would be formed assuming that planarity is maintained to ensure a better resonance therefore positive charge delocalization when compared to non-coplanarity.

The slight *enantiotopy* between the in silico and the crystallographic structure, which might be due to the inherent *snapshot* character of the X-ray methodology and the packing in the crystallographic structure, reflects a lesser energy of interaction, therefore smooth contouring of the latter structure, due to the steric hindering of the more constrained piperazine ring to the probe access (Figs 10 and 15).

Obviously, the possibility of alternate structures of the ofloxacin-cation complexes, in which water molecules, deprotonating effects of water or additional auxiliary bridging groups[13,15] might play a role, should be considered in either the neutral or in the charged forms of the ofloxacin complexes.[36–38] As expected, the highly solvated charged species form complexes in the region between the carboxyl and the carbonyl groups, as shown by the overlapping blue and red area (Fig. 13).

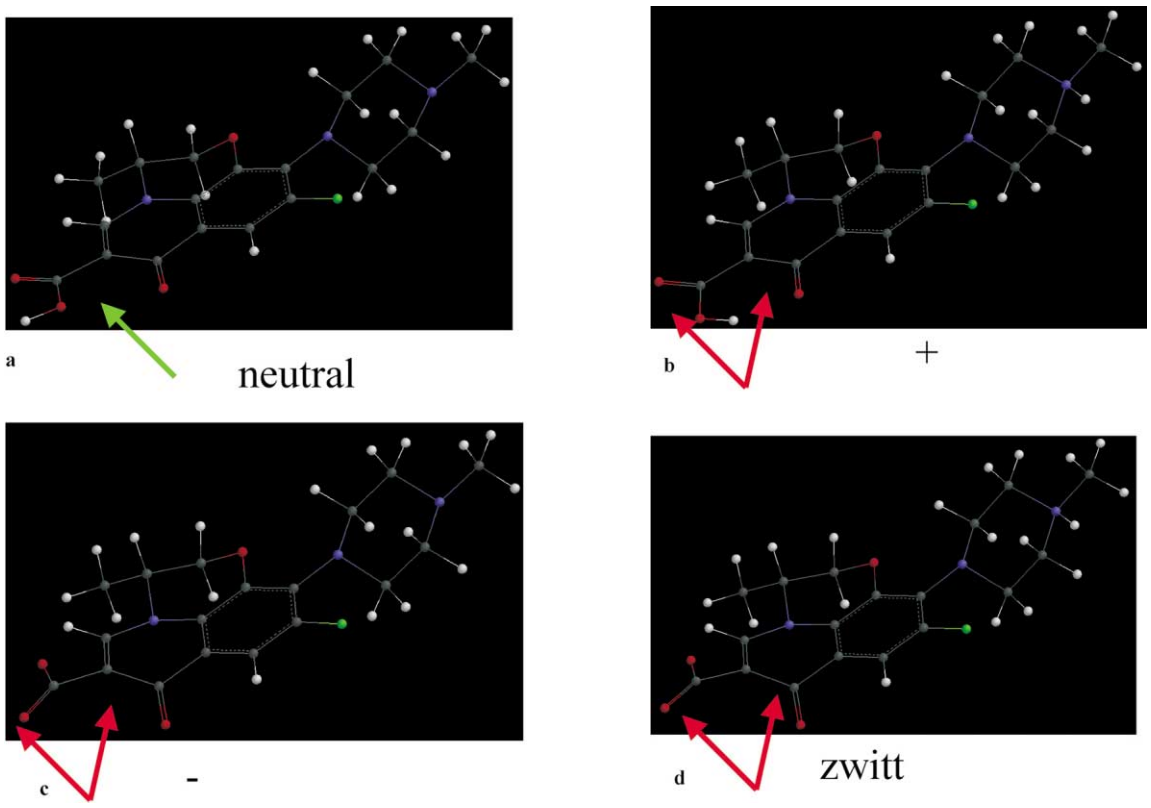

Figure 11. Ofloxacin in its neutral state (a): green arrow points to the planar carbonyl group. Ofloxacin in its positive (b), negative (c) and zwitterionic (d) forms: red arrows point to the outplane carboxyl group and the distorted pyrimidyne ring.

Discussion

Cytoplasmic membranes must be crossed by fluoroquinolones in order for them to reach their target, that is topoisomerase. An additional outer membrane, which can behave as a barrier to penetration, is also present in Gram-negative microorganisms. Porins and water-filled channels are present throughout the outer membranes, allowing two different biological environments to communicate. Hydrophilic fluoroquinolones may cross outer membranes through these porins by a passive diffusion process.[6] The driving force for this process is determined by the different drug concentrations inside and outside the cell. A passive diffusion process through the lipid bilayers of the outer membrane[39] can represent another entrance pathway for most hydrophobic members of the quinolone class. This particular behavior is termed fluoroquinolone self-promoted uptake, consisting of the formation of a chelation complex between divalent cations, such as magnesium, and the drugs. Chelation has been proposed to take place via the 4-ketone and 3-carboxylic acid groups of quinolones. This property seems to have a certain relevance in the cell entrance of these compounds.[39]

Therefore, the first step for ofloxacin passage into bacterial cells consists of the interaction between the drug and the cytoplasmic membrane. The dependence of the physico-chemical properties of ofloxacin on the pH of the environment may be reflected in ofloxacin biological interactions. Thus the different microforms at physiological pH should be taken into account in the interpretation of the protein binding of this drug, and in the drug passage through membranes via OmpF porins and/or passive diffusion. In fact, when a drug presents a log P_{oct} in the interval -0.3 to 1.8 (ofloxacin at pH 7.4), a compound shows the highest permeability through phospholipid membrane.[40] Under acidic conditions, ofloxacin assumes a certain hydrophilic characteristic, and this results in low permeation through the membrane (Fig. 6). This behavior is also determined by the positive charge of the drug at acid pH values. As shown by DSC experiments, a repulsion between ofloxacin and choline headgroups of DPPC bilayers takes place in acidic conditions.

By plotting the release data reported in Fig. 6 on a semi-log scale, straight lines were obtained (early step of drug release), showing first order release kinetics (data not reported). Thus, the partition of ofloxacin from the inner DPPC membrane aqueous phase into the phospholipid bilayer matrix should be one of the main factors controlling drug permeation. In the case of amphoteric fluoroquinolones, a larger biomembrane drug fraction tends to exist in the uncharged form of the drug at pH $\sim$7 if the 7-substituent becomes a weaker base, as in ofloxacin. For a number of fluoroquinolones, such as ofloxacin, an improved activity as a function of increased drug entry rate has been proposed.[6] Actually, the effect of charged/uncharged species of drugs on the cytoplasmic drug accumulation at equilibrium and on antibacterial efficacy is a more likely effect, rather than the relative abundance of the uncharged forms. It should be noted that efficacy and permeation are not only influenced by the physico-chemical properties of

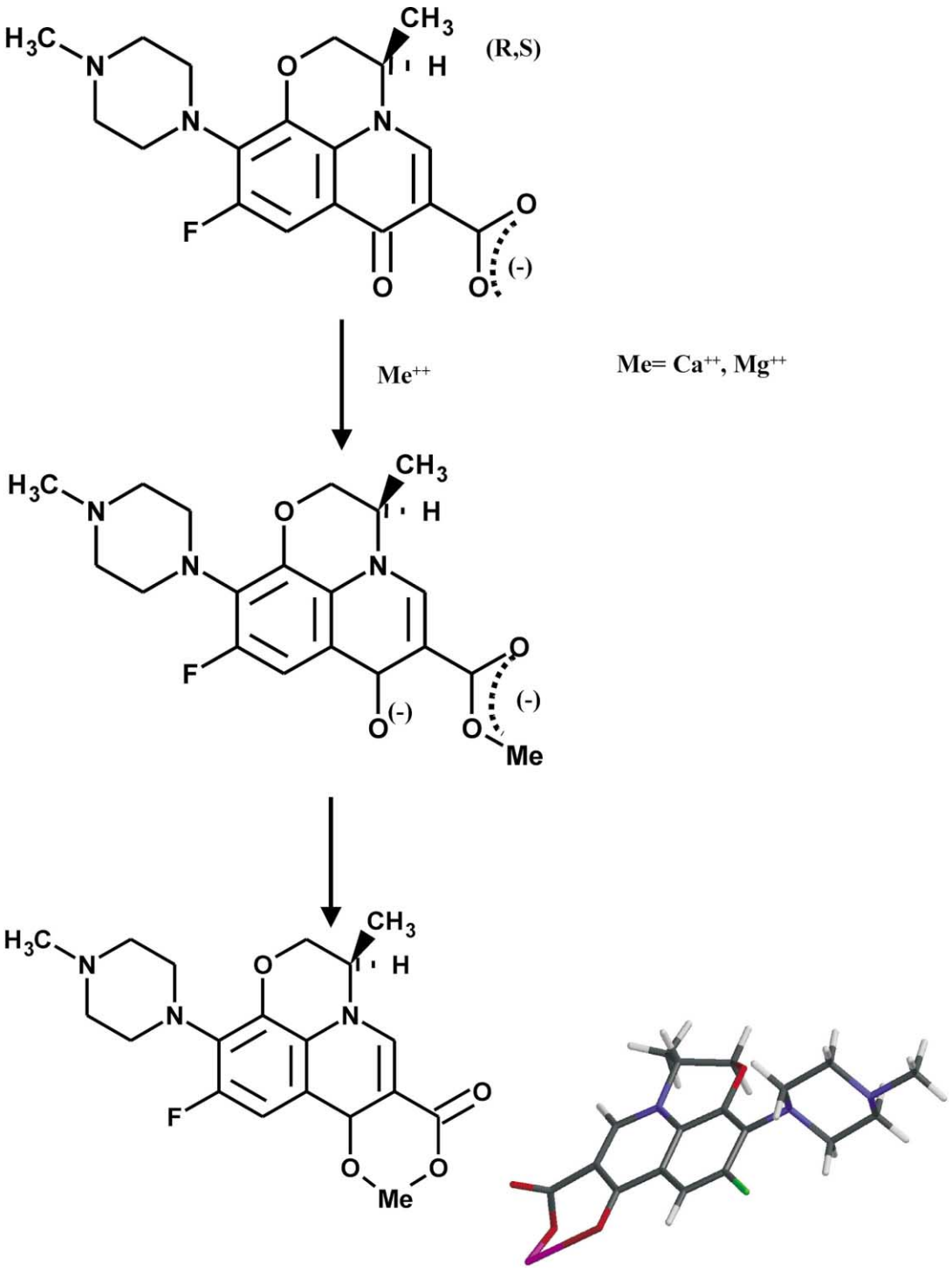

Figure 12. Cationic (Me^{++}) ofloxacin complex formation and its *tube* representation (bottom right).

drugs, but also by membrane properties. That is, there are membranes with much less fluid interiors, in which the diffusion of any solute is very slow, that is, the bacterial outer membrane bilayer.[41] This aspect was demonstrated by the more rapid permeation of ofloxacin through DMPC (fluid bilayer) than DPPC membranes (Fig. 6).

The slower diffusion of ofloxacin through membranes under acidic conditions demonstrates its accumulation and lysosomothropic properties. As a first approximation, only the uncharged forms of these agents diffuse through lipid bilayers, which are essentially impermeable to charged ionic drug forms. Therefore, ofloxacin (or similar drugs) may be entrapped within a membrane if a pH gradient exists between the inner and outer aqueous phase. Living bacteria maintain a proton driving force across the cytoplasmic membrane, and this provides the formation of a pH gradient. This is the case for *E. coli* cells, whose cytoplasmic pH (pH 6.1) is lower than the external pH (pH 7.5) by about 1.5 pH units.[42] Since equilibrium at a steady state is reached when the

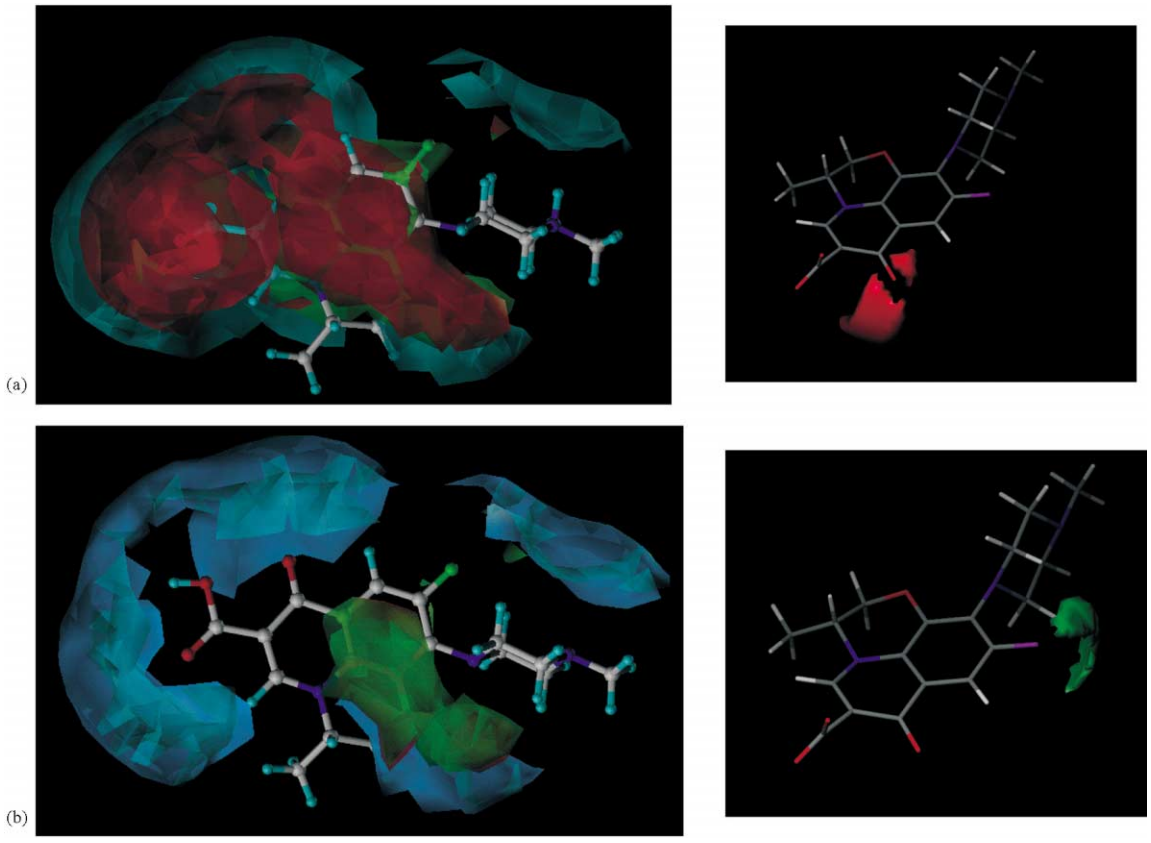

Figure 13. Interactions of ofloxacin in its neutral form (a) or internally compensated zwitterionic form (b) with calcium or magnesium cations. Blue: water (contour level: −3.0 to −7.0596 kcal/mol); red: Ca^{++}; green: Mg^{++} (contour levels for the two cations: −6 to −17.5617 for Mg^{++};−6 to −21.7244 kcal/mol for Ca^{++}. In the case of the neutral ofloxacin form, additional interactions are possible in a poorly solvated area (green contours) with morpholino oxygen and the piperazine nitrogen as the coordinating atoms (the interaction by the magnesium cation is stronger than that of the calcium cation). In the case of the zwitterionic ofloxacin form, the area of the stronger ionic interaction was found between the carboxyl and the carbonyl group, according to the location of the negative charges. The blue area surrounding the molecule shows the extended solvated area. The zwitterionic form is shown as an ensemble of the two possible charged forms. On the right a detail of the interaction at the expected carboxyl–carbonyl side (upper, contour level: −7.5 to −10.8 kcal/mol) and the additional interaction at the *unsolvated* area (down, contour level: −8.8 to −12.6 kcal/mol).

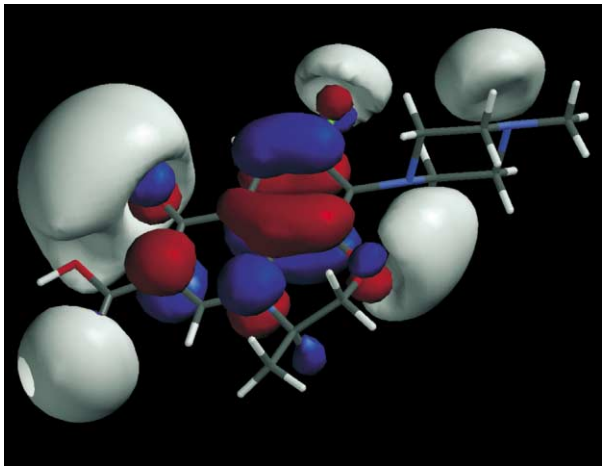

Figure 14. Fluorine and oxygen electron density at the *reactive* HOMO orbital. Electrostatic potential (elpot) is also displayed.

cytoplasmic and the external conditions of the uncharged form become equal, the total cytoplasmic concentration needs to be higher than in the medium. Namely, an accumulation is achieved (Fig. 16). This fact can justify the reduction of fluoroquinolone cell uptake in the presence of energy poisons.[43] In this case, blockage of the proton pump led to the loss of the transmembrane pH gradient, thus reducing the drug accumulation within cells. This phenomenon is particularly enhanced in the case of accumulation within lysosomes (lysosomothropic properties), which are characterized by marked acidic pH values (Fig. 16).

This hypothesis is consistent with data previously reported[6] with regard to drug cytoplasmic concentration. *N*-Methylation of piperazine decreases the pK_a of this group, leading to an increased sequestration of fluoroquinolones in the cytoplasm.[6] An increase in the activity without any change in affinity to the target was reported.[44] Namely, the MICs of most of the acidic quinolones are much lower than the concentrations needed to inhibit the target, suggesting that, as predicted, fluoroquionolones can accumulate in the cytoplasm.

DSC experiments showed that a certain interaction between ofloxacin and a bilayer membrane component occurred. These findings, in agreement with those found in literature,[25] suggest that part of the apparent drug uptake could be represented by simple binding to surface

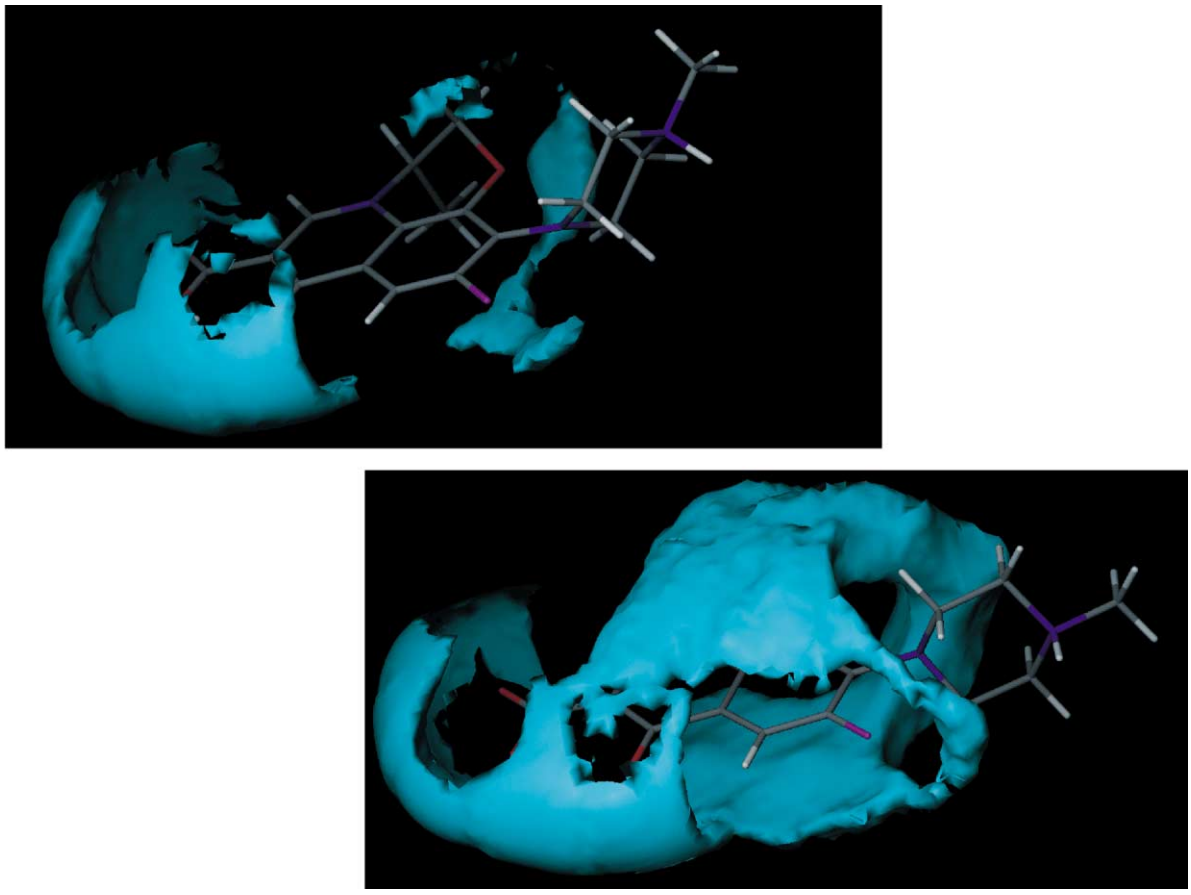

Figure 15. Upper: interaction of ofloxacin as in the Cambridge Structural Database (CSD code: SOYBEN)[28–30] with magnesium (contour level: −1.7 to −7.00 kcal/mol). Bottom: interaction of the in silico generated ofloxacin (lowest energy conformer) with magnesium (contour level: −1.7 to −7.00 kcal/mol). The maps are qualitatively quite similar. The *quantitative* difference is due to the piperazine ring restrictions (steric hindering) in the X-structure to the cation access. The steric hindering leads to a slighter interaction therefore to a less extended contouring area. See the Results and the Experimental for the explanation. Similar GRID maps were obtained using the Ca probe.[52]

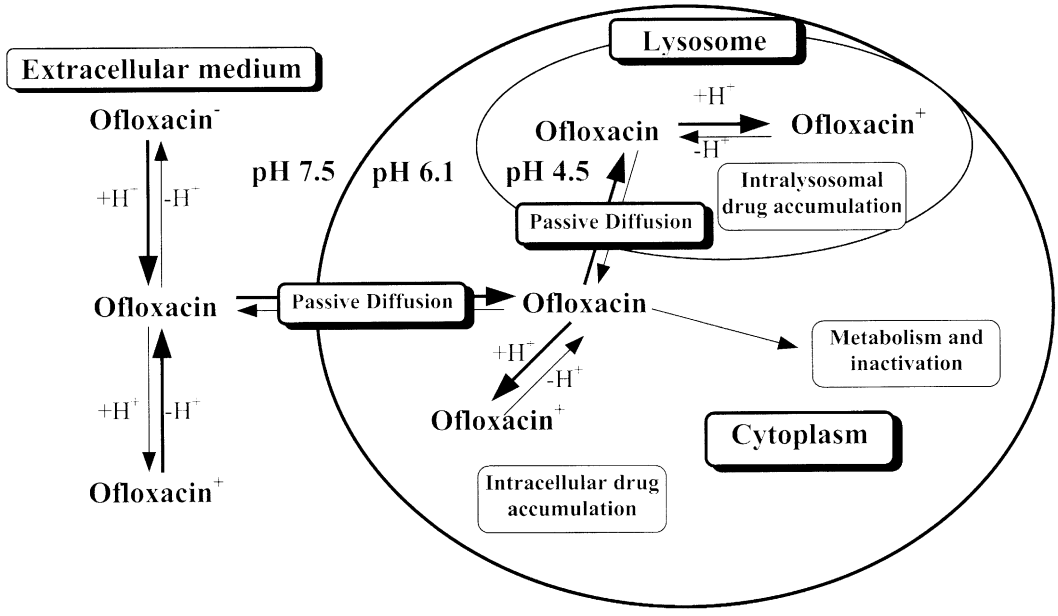

Figure 16. Schematic representation of the ofloxacin accumulation within bacterial cells and/or lysosomes. The bold arrows show the main direction of movement by which equilibrium is maintained.

membrane components. An interaction between a positively charged ofloxacin molecule and the head groups of DPPC phospholipids was observed (Fig. 3).

To resemble a negative cellular surface charge, DPPC/DPPA (8:2 molar ratio) biomembranes were prepared. DSC experiments demonstrated that ofloxacin is able to interact preferentially with the negatively charged component of a biological membrane through the piperazine moiety of its molecule. Our findings show that the drug protonation conditions play a fundamental role in the kind of interaction between ofloxacin and membrane bilayers. Therefore, fluoroquinolone interaction with bacterial membranes can play a role in drug accumulation, membrane permeability modulation and antibacterial effectiveness.[6]

The data herein reported support the hypothesis that the interior-negative Donnan potential across the bacterial outer membrane should produce an accumulation of fluoroquinolone in the periplasm with respect to that in the external medium. Equilibration across the cytoplasmic membrane will then increase the cytoplasmic concentration.

Besides passive diffusion of fluoroquinolones through the bacterial outer membrane and the cytoplasmic membrane, another entrance pathway is the porin route.[6] This pathway, present especially at the level of the outer membrane, can be used by charged species of a variety of antimicrobial agents, such as fluoroquinolones.[45] Porin-deficient mutants become more resistant to these agents.[45] In particular, loss of OmpF reduced the intracellular accumulation of hydrophilic fluoroquinolones such as ciprofloxacin and ofloxacin by 50%.[46] A certain importance for fluoroquinolone outer membrane penetration and for porin entrance seems to be the formation of complexes with divalent cations such as Mg^{++} and Ca^{++}, the self-promoted uptake.[25]

Ofloxacin, like other fluoroquinolones, can interact with the outer bacterial cell membranes by chelating lipopolysaccharide-associated Mg^{++}, thus creating hydrophobic patches on the cell surface. In these particular conditions, fluoroquinolones may penetrate the outer membrane of bacterial cells by means of the porin pathway and/or passive diffusion through hydrophobic patches, as a function of physico-chemical properties. In fact, diffusion through water-filled porins is influenced by the hydrophobicity of the compound, namely, the less hydrophobic an antibacterial drug is, the better it diffuses. While an inverse relationship occurs for the diffusion through a hydrophobic lipid bilayer.

Data, herein reported (Tables 3 and 4 and Fig. 8), suggest how the self-promoted penetration may work. Formation of Mg^{++}– and Ca^{++}–fluoroquinolone complexes (1:1 or 2:1 molar ratio as hypothesized) make the membrane bilayers more fluid, allowing a higher passive diffusion of solutes through it: the greater the membrane fluidity, the higher the solute permeability.

The complexing capacity of fluoroquinolones, such as ofloxacin, with respect to the divalent cations Mg^{++} and Ca^{++} can play a role not only in the permeation of the drug but also in the heterogeneous distribution between the internal and external environments of cells. The accumulation of ofloxacin, or other fluoroquinolones, within cells can be determined by the different divalent cation distribution, other than the pH value of the environment. In the extracellular environment, the formation of complexes between ofloxacin and Mg^{++} or Ca^{++} takes place and the cations may be subtracted from the outer layer of the membrane, allowing the formation of more diffusive hydrophobic zones, through which the solute may penetrate as a function of its physico-chemical properties. When ofloxacin reaches the cellular cytoplasm, a very low drug/Mg^{++} molar ratio is present; therefore, the amount of the drug is not sufficient to block and/or remove all Mg^{++} cations from the inner layer of the membrane, hampering the formation of a hydrophobic zone. As a consequence of this situation, the external and internal layer of membranes may show a different permeability with regard to the same diffusing compound (Fig. 17), leading to and/or favoring an accumulation of the drug. It should also be considered that the complex formation could also be influenced by pH, as demonstrated by molecular modeling studies. In fact, an acidic intracellular pH hampers the formation of divalent cation–ofloxacin complexes, because chelation requires a deprotonated carbonyl group. Furthermore, Mg^{++} complexes bearing a net positive charge can preferentially diffuse through porin channels, which usually prefer cations.

Ofloxacin accumulation profiles (Fig. 9) and biomembrane interaction experiments (Tables 3 and 4 and Fig. 8) show that the chelating properties of fluoroquinolones, particularly with respect to Mg^{++}, make the outer membrane more permeable and accessible. In fact, high concentrations of Mg^{++}, if present in the extracellular medium, inhibit the penetration and hence the activity of these antibacterial agents,[12] probably because a large excess of this divalent cation results in the chelation of most of the agents in the periplasm, thereby subtracting them from the equilibrium across the cytoplasmic membrane. This situation is much more evident for hydrophobic fluoroquinolones, which use the nonporin pathway for entry.[12]

The exposure of hydrophobic domains on the cell outer surface, due to the formation of fluoroquinolone–Mg^{++} complexes, can result in a higher susceptibility of the bacterial cells with regard to host immune system compounds, that is the complement. The chelation of Mg^{++} cations disorganizes the hydrophilic shell around bacteria cells, allowing it to bind to the host macromolecular substrate. In fact, the growth of *Klebsiella pneumonie* in the presence of sub-MICs of ciprofloxacin resulted in an increased binding of the complement component C3 to the bacterial surface.[47] Thus, fluoroquinolones can contribute to the elimination of pathogens by rendering them susceptible to components of the host immune system.

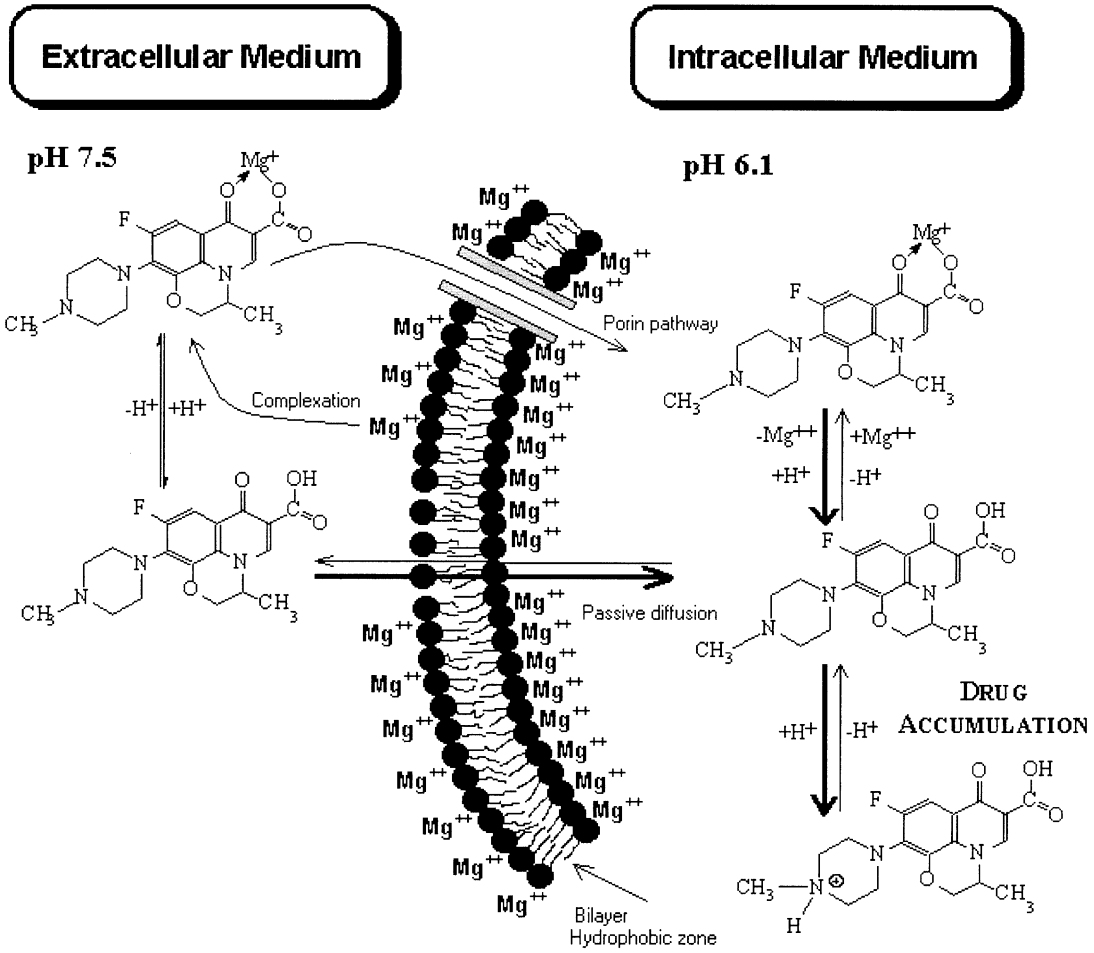

Figure 17. Schematization of the ofloxacin self-promoted uptake mechanism for cell entrance and possible heterogeneous distribution (accumulation). Chelation of Mg^{++} from the membrane surface by ofloxacin molecules elicits the formation of a hydrophobic bilayer patch, through which passive diffusion is favored. Mg^{++}–ofloxacin complexes can diffuse into cells through the porin pathway. Within cells, lower pH values hamper complex formation due to the protonation of the ofloxacin carbonyl group. Thus, the drug cannot diffuse back through the porin pathway. Furthermore, the higher intracellular Mg^{++} levels unfairly treat the formation of inner membrane leaflet hydrophobic domains, also hampering the back passive diffusion process. Therefore, ofloxacin may be intracellularly accumulated in relation to the proton and/or divalent cation concentration difference, when active expulsion mechanisms do not occur (active pump-mediated resistance).

The fluoroquinolone behavior, as a function of pH value and divalent cation concentration, could also be the explanation for the fact that amphoteric fluoroquinolones are more active against Gram-negative than Gram-positive bacteria. Another reported observation[48] consistent with this model is the great effect of the external pH on the efficacy of amphoteric fluoroquinolones against *E. coli.*

Therefore, these aspects should be studied and fully elucidated, since intracellular infections are of importance not only for obligatory intracellular parasites but also for facultative parasites. The absolute rate of fluoroquinolone entry is important in modulating antibacterial efficacy, but in a number of cases, such as ofloxacin, equilibrium distribution plays a more important role.

Conclusion

Synergy between theoretical and experimental data has proven to be a very powerful tool to elucidate and/or open new gates not only in strictly chemistry related topics[49] but also in drug delivery. The experimental results are in agreement with computational ones and both demonstrate that the ofloxacin entrance pathway through membranes, in the same way as other fluoroquinolones, can be explained as a result of simple, passive diffusion through the porin channels and membrane bilayers and its distribution triggered by the Donnan potential, pH gradients and divalent cation chelation (Mg^{++} and Ca^{++}).

Experimental

Calcein was obtained from Sigma Chemical Co. (St. Louis, USA). DPPC, DMPC and DPPA were obtained from Fluka Chemical Co. (Buchs, Switzerland). Before use, the lipid purity (greater than 99%) was assayed by two-dimensional thin-layer chromatography on silica gel plates (E. Merck, Darmstadt, Germany).[9] Ofloxacin was a gift from Sigma-Tau S.p.A. (Pomezia, Italy). The purity of this drug was greater than 99.5% as assayed by HPLC analysis. Double distilled water was used. All other materials and solvents were analytical grade (Carlo Erba, Milan, Italy).

Octanol and different aqueous phases (buffers at different pH values ranging from 4 to 10) were used to determine the partition coefficient. The organic solvent and the aqueous buffer were presaturated with each other by thorough stirring for 24 h at 37 °C in a glass flask. Octanol (10 mL) was layered onto the aqueous buffer (10 mL) and the drug (1 mg) was then added. This mixture was stirred (1000 rpm) for 3 h at 37 °C. Phase separation between octanol and buffer was obtained by centrifugation at 3000*g* for 15 min. Ofloxacin amounts in the various phases were spectrophotometrically determined at 286 nm (Uvikon 820, Kontron Instruments, Zurich, Switzerland). A straight calibration line as a function of the various pH values was used. The partition coefficient (log P) is expressed as the logarithm of the ratio between the amount of ofloxacin in the organic phase and that in the aqueous phase. Drug recovery was always higher than 98%.

Biological membrane models were prepared as elsewhere reported.[9] Briefly, phospholipid components were dissolved in chloroform in a round-bottomed flask. Phospholipid films formed on the wall of the vessel after evaporation of the solvent at 35 °C with a rotary evaporator. The films were stored for 12 h in a Buchi T-50 under high vacuum at 40 °C. Lipid films (10 mg) were hydrated by adding 200 μL of aqueous phase (isotonic buffer at different pH values, or saline solution with Mg^{++} or Ca^{++} at different concentrations) containing a certain amount of the drug in order to obtain the desired molar fraction. The flask was alternatively vortexed for 4 min and warmed in a water bath at 55 °C for 5 min. The procedure was repeated four times. Finally, the vesicle suspension was left at room temperature for 1 h to anneal the bilayer structure. All the procedures were carried out under nitrogen to prevent oxidation of the lipid.

The resulting lipid vesicles were extruded by means of a stainless steel extrusion device (Lipex Biomembranes, Vancouver, BC, Canada) equipped with 10-mL water-jacketed 'thermobarrel' connected to a thermostat, which allowed extrusion at 55 °C.[50] In order to obtain large unilamellar and/or oligolamellar vesicles, the extrusion procedure consisted of ten passages of the lipid vesicle suspension through 400 nm polycarbonate filters.

The various samples (40 μL containing ~2 mg of lipid material) were sealed in an aluminum pan and submitted to differential scanning calorimetry (DSC) analysis. The calorimetric experiments were performed with a Mettler DSC 12E (Mettler-Toledo, Greifensee, Switzerland) equipped with a Haake D8-G (Haake Mes-Technik, Karlsruhe, Germany). Indium was used to calibrate the instrument, that is, temperature scale and enthalpy changes (ΔH). The detection system was a Mettler Pt100 sensor. The sensor presented a thermometric sensitivity of 56 μV/°C, a calorimetric sensitivity of about 3 μV/mW and a noise level lower than 60 nV (<20 μW) peak to peak. The baseline reproducibility was <3 μV (<1 mW). Each DSC scan presented an accuracy of ±0.4 °C with a reproducibility and a resolution of 0.1 °C. The reference was an aluminium pan containing 40 μL suitable isotonic buffer. Reference and sample pan masses were always matched to within 5.3% total mass, and usually to within 1.8%. The various samples were submitted to heating and cooling cycles (three times), in the temperature range 20–60 °C at a scanning rate of 1 °C/min. The data from the first scan were always discarded to avoid mixing artifacts. The necessity of periodic recalibration of the system over the course of these experiments may obviate direct comparison between groups of results. For this reason, a control sample, containing the basic phospholipid preparation without any drug, accompanied each set of studies. The endotherm obtained during the second scan of this control sample was used as a reference template for analysis of the accompanying experiments. ΔH values were calculated from peak areas by a Mettler TA89A system software (version 4.0).

Ofloxacin permeability through DPPC biomembranes was evaluated following drug leakage as a function of time. The untrapped drug was removed from DPPC vesicles by gel permeation chromatography. An aliquot (1 mL) of DPPC vesicles was loaded into a Sepharose 4B fine column (1.5-cm diameter, 50-cm length; Pharmacia, Upsala, Sweden). The gel permeation was carried out at room temperature with a suitable isotonic buffer as the mobile phase. The flow rate was set at 250 μL/min with a peristaltic pump and fractions of 3 mL were collected. The detection was carried out both at 550 nm, to assay sample turbidity, and at drug λ_{max} 286 nm to follow free drug elution. The drug-loaded DPPC biomembranes prepared at different pH values were dispersed in 200 mL of pH 7.4 isotonic phosphate buffer and incubated at 37 ± 0.1 °C (Haake F3-R) under gentle mechanical stirring. At appropriate intervals, 2 mL portions of the various DPPC biomembrane suspensions were withdrawn, transferred into Whatman VectaSpin3 ultrafiltration membrane cones (mol. wet. cut off 30,000; Whatman International Ltd, Milano, Italy) and centrifuged at 10,000*g* for 5 min. The filtrate containing the released drug was assayed spectrofluorimetrically: λ_{ex} 286, λ_{em} 454, $r = 0.9997$. No drug-membrane association was observed.

To evaluate the influence of ofloxacin in the presence of Mg^{++} cations on the DPPC permeability, the calcein leakage from vesicles was studied. Calcein-loaded DPPC vesicles were prepared in the presence of Mg^{++} at a concentration of 7.2 or 348 mg/L, and any untrapped fluorescent probe was separated by gel-permeation chromatography as previously described. Calcein release was evaluated both in the presence and in the absence of ofloxacin. Vesicle suspension aliquots (1 mL) were withdrawn and spectrofluorimetrically assayed for the fluorescent probe. Calcein presented λ_{ex} 493 nm and λ_{em} 518 nm. Ofloxacin was added to a calcein-loaded DPPC vesicle suspension at a concentration of 0.25 mg/mL. The ofloxacin–Mg^{++} molar ratios were 2:1 and 1:24 for Mg^{++} concentrations of 7.2 and 348 mg/L, respectively.

To evaluate the complexing capacity of ofloxacin, drug fluorescence spectra were recorded as a function of the

presence of different cations (i.e., Na^+ K^+, Mg^{++} and Ca^{++}) at different concentrations. Ofloxacin was solubilized in distilled water at a concentration of 10 ng/mL. Fluorescence spectra were recorded with a Hitachi model F-2000 spectrofluorimeter (Hitachi Ltd., Tokyo, Japan). Various aqueous solutions without the drug were used as references to evaluate the fluorescence background. The excitation and emission wavelength were 286 and 454 nm, respectively.

Ofloxacin accumulation was measured as elsewhere reported.[51] *E. coli* (ATCC 25922) was incubated under continuous shaking at 37 °C up to an $A_{660} = 0.7$–0.8. Bacteria were harvested, washed and concentrated 20-fold in 50 mM sodium phosphate buffer pH 7.0. Ofloxacin was added to this cellular suspension to a concentration of 10 μg/mL. At predetermined time intervals, 500 μL was withdrawn, poured into a microcapped centrifuge tube containing 1 mL chilled 50 mM sodium phosphate buffer and centrifuged at 4 °C. Pellets were washed twice and resuspended overnight at room temperature in 1 mL 0.1 M glycine hydrochloride (pH 3.0), allowing cell lysis. Cell debris were removed by centrifugation (10,000*g*) at 25 °C for 5 min. Proteins were precipitated by treating various samples with acetonitrile. The amount of ofloxacin in the supernatant was determined by HPLC analysis.

The chromatographic apparatus was a Hewlett-Packard model 1050 system (Hewlett-Packard, Avondale, PA, USA), equipped with a Hewlett-Packard 1046A fluorescence detector operating at excitation and emission wavelengths of 286 and 454 nm, a Rheodyne 7125 loading injection valve (Rheodyne, Cotati, CA, USA) with a 20-μL loop and a Hewlett-Packard 3395 reporting integrator. Chromatographic separation was carried out at room temperature with a 5-μm particle size Hypersil C_{18} cartridge column (125 by 4.6 mm inner diameter) obtained from Alltech (Milan, Italy) and equipped with a direct-connect guard column. Ofloxacin was eluted with a mobile phase consisting of acetonitrile and buffer (2 g of sodium acetate, 2 g of sodium citrate, 1 mL of triethylamine in 850 mL of HPLC water, pH 4.5) mixture (40:60, v/v) at a flow rate of 1 mL/min. The eluent was filtered through a 0.2-μm pore size Teflon membrane (Spartan-3 Schleicher & Schuell, Keene, NH, USA) and degassed by ultrasonication prior to use. For biological samples, enoxacin was used as internal standard. The lower detection of ofloxacin in biological samples was 5 ng/mL. HPLC reproducibility was determined by repetitive analysis of biological samples spiked with a known amount of ofloxacin. The intra-assay and inter-assay C.V. were 0.86 and 1.07%, respectively. No interference from the other components present in the various samples was observed during HPLC analysis.

The molecular modeling studies were carried on a SGI R5000 workstation operating under IRIX 6.5.+ performed using quantum mechanical methods (ab initio/semi-empirical) as implemented in the Spartan 5.1.3. suite[26] and the software GRID (versions 17, 19 and 20).[16–18] Supporting evidence on the consistency of the in silico ofloxacin structure, both in terms of root mean square (rms) and strain energy values, were collected by a comparison with the CSD (Cambridge Structural Database) entry (code: SOYBEN) using the softwares SYBYL[32] and the QXP/FLO module as implemented in FLO01.[33,34] The latter uses an Amber force field implemented to provide attractive energetics for atoms of the same type, while minimizing individual internal energies. FLO01/QXP was used to flexibly dock each molecule to the template CSD structure.[33,34] In order to further test the reliability of the in silico generated structure a missing piperazine rms value was also calculated by SYBYL.[32] The CSD structure[28–30] was cleaned from the perchlorate anion to avoid a puzzling effect because of exceeding contouring (Fig. 10). GRID was used to calculate intermolecular interaction energies and map the putative sites of calcium and magnesium complex formation with ofloxacin. The coordinates of each structure were carefully checked, and the default parameters of the GRID force field as well as the default GRID spacing of 1 Å were used.[16–18] The GRID methodology approach gives a large volume of good attractive interactions, the so called 'GRID-map', between the molecule and the probe. In the Grid program, a three-dimensional grid surrounds the target molecule. The versions 17, 19 and 20 of GRID[16–18] work like previous versions by computing the interaction energy of a probe at every Grid point on an orthogonal matrix of points around the molecule of interest. The interaction energy between a probe and each atom of the target was then calculated for each Grid point, and this calculation generated one Grid map. By contouring the Grid map at various energy levels, one can display favorable interaction areas between the molecule and the probe as in Figures 13 and 14.

There is a GRID 'flexibility' option in the versions 17, 19 and 20[16–18] which allows the possible rotatable chains in the test molecule to move in response to the probe. They move toward the probe when there is attraction and away from it when there is repulsion, thus simulating the response of the target to a change in its environment. This flexibility option was turned *off* in this study as there are no differences in the map when the option was turned *on* as a consequence of the quite rigid ofloxacin structure. Within a certain tolerance limit of flexibility, different conformations do not affect the GRID maps.[16–18] The same reason can account for the qualitatively similar maps between the crystallographic (E_{cryst})[28–30] and the in silico (E_{silico}) structures, which only differ in the energy of interaction (E_{cryst}: 7 kcal/mol; E_{silico}: 16 kcal/mol). The steric hindering due to the more folded piperazine ring in the CSD structure restricts the probe access and therefore the strength of the interaction (Fig. 15).

GRID probes are chemical groups such as methyl, aliphatic hydroxyl, NH_3^+ amine, water and divalent cations and the interaction energy at each Grid point is calculated between the chosen probe and every atom of the molecule. The probes are characterized by their steric, electrostatic and hydrogen-bonding properties and by their hybridization.[16–18] In this study probes were selected in order to mimic chemical groups which are present in the supposed complex.

The GRUB datafile of GRID was modified according to Fraga et al.[52] to allow the GRID recognizing of a neutral covalent complex so avoiding any interaction with the cation (Ca^{++} or Mg^{++} probe) at the carboxyl–carbonyl site to study the hypothesized additional interaction at the *heterocyclic* side. Fraga's[52] parameters gave the most reliable result when compared to other literature sources or de novo theoretical calculations. Starting structures were built using the tools and the fragment library present in Spartan (version 5.1.3)[26] by assigning the *R* stereochemistry to the morpholino stereogenic center of the ofloxacin molecule. The *R* enantiomer was considered, assuming similar entrance and caging mechanisms of *S* enantiomer.[53] Spinput (shortcut for Spartan Input) files[26] were exported as pdb files. In order to determine the lowest energy conformation of various charged and uncharged ofloxacin forms, a Montecarlo conformational search was carried out by molecular mechanics using the MMFF94 (Merck Molecular Force Field) as implemented in Spartan.[26] The torsion ranges of all the ofloxacin rotatable bonds, except the methyl substituents single bonds as in the piperazino and morpholino rings which are 'negligible', were scanned within 0–360° (360/36, 10° increments). Torsion ranges of the *improper* cyclic single bonds as in the piperazino and morfolino cycles were also scanned using the same settings to determine whether the oxygen and the nitrogen atom geometry is planar/not planar. A sp3 oxygen and a sp2 nitrogen were finally calculated (Fig. 11). A formal charge of +1 (assuming a localization on the pyrimidine nitrogen) was considered in the conformational analysis of the calcium and magnesium complexes, thus obtaining 11.025 conformers for all of the calculated ofloxacin structures (both complexed and free). Following the conformational search, the structure of the lowest energy conformers were re-minimized using the semi-empirical PM3 method, which was used to calculate the heat of formation (ΔH), being itself parameterized on heat of formation values. All the settings were default values. The ab initio STO-3G and 3-21G* basic sets as implemented in Spartan[26] were used to check the reliability of the PM3 results and the intramolecular hydrogen bond between the carboxyl and the carbonyl groups in the pirymidine ring of ofloxacin. The polarizability and hyperpolarizability functions, which are specific for hydrogen bonds, were included in the calculation.[26] The AMBER* FF, as implemented in the Macromodel (version 6.5),[27] was used to verify the MMFF94 result. Default options were used with the Polak-Ribiere conjugate gradient minimization (PRCG) allowing for a maximum of 500 iterations per structure, until a gradient of 0.05 kJ/mol-Å was reached.

Electrostatic potentials (Elpots) and HOMO (Fig. 15) calculations were carried out using single point energy at the PM3 level[26] on a structure minimized at the same level. The calculated values were in agreement with ab initio single point energy calculations (3-21G*) on a structure minimized at the STO-3G level.[26]

Acknowledgements

We thank Dr. Jenny Glusker (The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, PA, USA), Dr. Robert A. Scherrer (3M Pharmaceuticals, Chemistry-Drug Discovery, St. Paul, MN, USA) and Professor Giuseppe Arena (Department of Chemistry, University of Catania) for their helpful discussion and suggestions. We are grateful to Professor P. Goodford (University of Oxford, Laboratory of Molecular Biophysics, The Rex Richards Building, South Parks Road, Oxford OX1 3QU, UK) and Dr. Colin McMartin (Thistlesoft, PO Box 227, Colebrook, CT 06021, USA) for providing the GRID (Version 17,19 and 20)[16–18] and the FLO01 software,[33,34] respectively and their illuminating conversation.

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