Characterization of the Interactions between Fluoroquinolone Antibiotics and Lipids: a Multitechnique Approach

Hayet Bensikaddour,* Nathalie Fa,* Ingrid Burton,† Magali Deleu,‡ Laurence Lins,§ André Schanck,¶ Robert Brasseur,¶ Yves F. Dufrêne,† Erik Goormaghtigh,‖ and Marie-Paule Mingeot-Leclercq*

*Université Catholique de Louvain, Faculty of Medicine, Unité de Pharmacologie Cellulaire et Moléculaire, Brussels, Belgium; †Université Catholique de Louvain, Faculty of Agronomy, Unité de Chimie des Interfaces, Louvain-la-Neuve, Belgium; ‡Faculté Universitaire des Sciences Agronomiques de Gembloux, Unité de Chimie Biologique Industrielle, and §Centre de Biophysique Moléculaire Numérique, Faculté Universitaire des Sciences Agronomiques de Gembloux, Gembloux, Belgium; ¶Université Catholique de Louvain, Louvain-la-Neuve, Faculty of Sciences, Unité de Chimie Structurale et des Mécanismes Réactionnels, Belgium; and ‖Université Libre de Bruxelles, Faculty of Sciences, Unité de Structure et Fonction des Membranes Biologiques, Brussels, Belgium

ABSTRACT Probing drug/lipid interactions at the molecular level represents an important challenge in pharmaceutical research and membrane biophysics. Previous studies showed differences in accumulation and intracellular activity between two fluoroquinolones, ciprofloxacin and moxifloxacin, that may actually result from their differential susceptibility to efflux by the ciprofloxacin transporter. In view of the critical role of lipids for the drug cellular uptake and differences observed for the two closely related fluoroquinolones, we investigated the interactions of these two antibiotics with lipids, using an array of complementary techniques. Moxifloxacin induced, to a greater extent than ciprofloxacin, an erosion of the DPPC domains in the DOPC fluid phase (atomic force microscopy) and a shift of the surface pressure-area isotherms of DOPC/DPPC/fluoroquinolone monolayer toward lower area per molecule (Langmuir studies). These effects are related to a lower propensity of moxifloxacin to be released from lipid to aqueous phase (determined by phase transfer studies and conformational analysis) and a marked decrease of all-*trans* conformation of acyl-lipid chains of DPPC (determined by ATR-FTIR) without increase of lipid disorder and change in the tilt between the normal and the germanium surface (also determined by ATR-FTIR). All together, differences of ciprofloxacin as compared to moxifloxacin in their interactions with lipids could explain differences in their cellular accumulation and susceptibility to efflux transporters.

INTRODUCTION

Since their discovery in the early 1960s, the quinolone group of antibacterials has generated considerable clinical and scientific interest including the development of the second-generation quinolones like ciprofloxacin. These wide spectrum drugs are characterized by the introduction of fluor into position C-6 on the molecule. Progressive modifications in their chemical structure have resulted in improved breadth and potency of in vitro activity and pharmacokinetics (1). The most significant developments have been enhancement of the therapeutic potential of fluoroquinolones thanks to liposomal encapsulation (2–4) and improved anti-Gram-positive activity of the newer compounds like moxifloxacin (5).

Due to their ability to accumulate inside phagocytes (1,6–8), fluoroquinolones are also useful for eliminating facultative intracellular pathogens that resist phagocytic death. We recently showed that fluoroquinolones accumulate in macrophages and show activity against a large array of intracellular organisms including *Listeria monocytogenes* and *Staphylococcus aureus* (9). Quite significant differences among closely related derivatives have been observed with the following ranking in cellular accumulation and intracellular activity: ciprofloxacin < levofloxacin < garenoxacin < moxifloxacin (9). So far, to our knowledge, this has not received satisfactory explanation.

Characterization of fluoroquinolones uptake by eukaryotic cells suggested that both passive diffusion and active transport systems are involved. The transbilayer diffusion of fluoroquinolones has been demonstrated (10) and our group reported that ciprofloxacin, but not moxifloxacin, is subject to constitutive efflux in J774 macrophages through the activity of an MRP-related transporter (11).

Drug/lipid interactions can modulate not only translocation of the drug through the natural membranes but also its interaction with efflux proteins (12,13). In this respect, it is well known that 1), substrates have to be transported from the lipid bilayer to the transporter protein before a capture mechanism of the drug by the inner leaflet of the cytoplasmic membrane (14); and 2), the activity of transporter is critically dependent on the surrounding lipid bilayer environment (15,16), which may be modified by drugs.

In view of the critical role of lipids for the drug cellular uptake and differences observed for two closely related

Submitted June 15, 2007, and accepted for publication October 3, 2007.

Hayet Bensikaddour and Nathalie Fa contributed equally to the work.

Address reprint requests to Marie-Paule Mingeot-Leclercq, Tel.: 32-2-764-7374; E-mail: mingeot@facm.ucl.ac.be.

Nathalie Fa's present address is Campden & Chorleywood Food Research Association Group, Chipping Campden, Gloucestershire, GL55 6LD, UK.

Editor: Petra Schwille.

0006-3495/08/04/3035/12 $2.00

doi: 10.1529/biophysj.107.114843

compounds, ciprofloxacin and moxifloxacin (Fig. 1), we investigated the interactions of these two fluoroquinolones with lipids, using an array of complementary techniques. For both ciprofloxacin and moxifloxacin, atomic force microscopy (AFM) reveals an erosion of dipalmitoylphosphatidylcholine (DPPC) domains within dioleoylphosphatidylcholine (DOPC) fluid phase while Langmuir studies show a condensing effect. Further molecular studies show that fluoroquinolones can 1), exchange from lipids to aqueous phases (phase transfer and molecular modeling studies); 2), decrease the all-*trans* conformation of lipid acyl chain (attenuated total reflection Fourier transform Infra-Red (ATR-FTIR)); and 3), increase the lipid disorder (ATR-FTIR). When the effects of the two fluoroquinolones are compared, it clearly appears that moxifloxacin has a higher condensing effect related to a lower propensity to be released in the aqueous phase from lipid monolayer and to a higher ability to decrease the all-*trans* conformation of lipid acyl chain without marked effect in lipid-chain orientation. All together, differences of ciprofloxacin as compared to moxifloxacin in their interactions with lipids can be related to differences in their cellular accumulation and therefore activity against intracellular bacteria.

MATERIAL AND METHODS

Materials

Dioleoylphosphatidylcholine (DOPC) and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma (St. Louis, MO). Ciprofloxacin; microbiological standard, potency 85,5%, MW = 331.34 g/mol and moxifloxacin; microbiological standard, potency 91%, MW = 401.4 g/mol) were obtained from Bayer Healthcare AG (Leverkusen, Germany). All other reagents were from E. Merck (Darmstadt, Germany).

Fluoroquinolone assays

Ciprofloxacin assay

Ciprofloxacin content was determined by a fluorimetric method (λ_{ex}, 275 nm; λ_{em}, 450 nm, using a model No. LS-30 Fluorescence Spectrophotometer; Perkin-Elmer, Beaconsfield, UK) as described previously (11). Under these conditions, our assay had a lower detection limit of ~5 ng/ml, a linearity ($r^2 \geq 0.99$) up to 200 ng/ml, and an intraassay reproducibility of 97%.

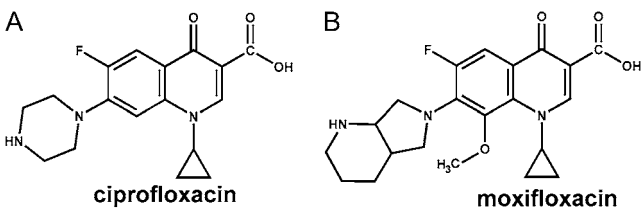

FIGURE 1 (*A*) Structural formula of ciprofloxacin (3-quinolinecarboxylic acid, 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-(9CI)) and (*B*) moxifloxacin (3-quinolinecarboxylic acid, 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-((4aS,7aS)-octahydro-6H-pyrrolo(3,4-b)pyridin-6-yl)-4-oxo-(9CI)).

Moxifloxacin assay

Fluorescent assay based on the same technique as that used for ciprofloxacin but using $\lambda_{ex} = 298$ nm and $\lambda_{em} = 504$ nm; lower limit of detection, 5 ng/ml; linearity up to 450 ng/ml ($r^2 \geq 0.99$); intraassay reproducibility, 98%.

Preparation of liposomes (MLVs, SUVs)

Lipid vesicles were prepared as described previously (17). Briefly, appropriate lipids were mixed in $CHCl_3/CH_3OH$ 2:1 (v/v), evaporated under nitrogen flow, and desiccated under vacuum for at least 4 h. The dried films were then resuspended at room temperature from the walls of the glass balloon by vigorous vortexing in aqueous buffer. Lipid in suspension flushed with nitrogen were kept in a water bath for 1 h at 37°C for pure DOPC or 45°C for liposomes containing DPPC. This procedure yields multilamellar vesicles (MLVs). The small unilamellar vesicles (SUVs) consisting of DOPC/DPPC (1:1 mol) were prepared from MLVs. The preparation, cooled down by an ice bath, was sonicated to clarity five times for 2 min each using a Fisher Bioblock Scientific 750 W sonicator (Avantec, Illkirch, France) set at 35% of the maximal power, and a 13-mm probe. The SUVs preparation was then filtered on 0.2 μm Acrodisc filters (Ann Arbor, MI) to eliminate titanium particles. The concentration of lipids, the nature of the buffer, and the lipid/drug ratio was adjusted for each type of experiment.

AFM imaging

Mica sheets were heated 1 h before fusion at 60°C and cleaved to obtain a flat and uniform surface. The SUV suspension of DOPC/DPPC (1:1) (10 mg lipids/ml; buffer 10:100:3 mM, pH 7.4), was put into contact with the mica surface for 45 min at 60°C and the sample was slowly cooled back to room temperature to prevent thermal shock. The excess of SUVs was then eliminated by four-times rinsing with a Tris/NaCl 10:100 mM buffer, pH 7.4. The sample was installed on the microscope without dewetting. The liquid meniscus was completed with the same buffer or solution containing 0.2 mM fluoroquinolones. All AFM measurements were carried out at room temperature in contact mode using an optical detection system equipped with a liquid cell (Nanoscope IV; Digital Instruments, Santa Barbara, CA). Topographic images were taken in the constant-deflection mode using oxide-sharpened microfabricated Si_3N_4 cantilevers (Park Scientific Instruments, Mountain View, CA) with typical curvature radii of 20 nm and spring constant of 0.01 N/m. Scan rate ranging from 4 to 6 Hz were tested. The applied force was maintained as low as possible (<1 nN) during the imaging. All images were flattened.

Partition of fluoroquinolones—phase transfer assay between aqueous and lipid phases

For the phase transfer assays, 1 ml of the water phase (Tris pH7.4, 10mM), containing the fluoroquinolone (1 μM), was mixed by vortexing for 30 s, to 1 ml of organic phase ($CHCl_3$), with or without lipids (Egg yolk phosphatidylcholine (PC)) from a lipid/drug ratio of from 0.1:1 up to 50:1. Both phases were decanted overnight at 4°C. The fluoroquinolone recovered from the water, interfacial, and organic phases were quantified by fluorimetry (model No. LS30 fluorimeter; Perkin-Elmer).

Interaction between fluoroquinolones and a model membrane by molecular modeling: the IMPALA procedure

The ciprofloxacin or moxifloxacin molecule was inserted into an implicit simplified bilayer using the IMPALA method described previously (18). This method simulates the insertion of any molecule into a bilayer by adding energy restraint functions to the usual energy description of molecules. The lipid bilayer was defined by $C_{(z)}$, which represents an empirical function

describing membrane properties. This function is constant in the membrane plane (x and y axes), but varies along the bilayer thickness (z axis) and, more specifically, at the lipid/water interface corresponding to the transition between lipid acyl chains (no water = hydrophobic core) and the hydrophilic aqueous environment,

$$C_{(z)} = 1 - \frac{1}{1 + e^{\alpha(z - z_0)}},$$

where α is a constant equal to 1.99; z_0 corresponds to the middle of polar heads; and z is the position in the membrane.

Two restraints were imposed to simulate the lipid membrane: the bilayer hydrophobicity (E_{pho}); and the lipid perturbation (E_{lip}).

The hydrophobicity of the membrane is simulated by E_{pho},

$$E_{pho} = -\sum_{i=1}^{N} S_{(i)} E_{tr(i)} C_{(zi)},$$

where N is the total number of atoms; $S_{(i)}$ the accessible surface to solvent of the i atom; $E_{tr(i)}$ its transfer energy per unit of accessible surface area; and $C_{(zi)}$ the $_{zi}$ position of atom i.

The perturbation of the bilayer by insertion of the molecule was simulated by the lipid perturbation restraint (E_{lip}),

$$E_{lip} = a_{lip} \sum_{i=1}^{N} S_{(i)} (1 - C_{(zi)}),$$

where a_{lip} is an empirical factor fixed at 0.018 kcal mol^{-1} Å^{-2}.

The environment energy (E_{env}) applied on the drug that inserts into the membrane becomes equal to

$$E_{env} = E_{pho} + E_{lip}.$$

Restraint plots

Diagrams showing the restraint values versus the angle between the helix axis and the bilayer normal or versus the penetration of the mass center are obtained as follows: for each degree (angle) or for each 1/10 Å (penetration), the lowest restraint value obtained during the Monte Carlo simulation is taken. All the points are then joined to generate a profile of the simulation.

Calculations are performed on an Intel Pentium 4, CPU 3.80 GHz, 4.00 GB of RAM. The calculation software has been developed at the CBMN (Gembloux, Belgium). Molecular graphs were drawn using WinMGM 1.0 (Ab Initio Technology, Obernai, France) and Sigmaplot 5.0 (SPSS, Chicago, IL) was used for data analysis.

Surface-pressure isotherms of lipid monolayer—Langmuir trough experiments

An automatically controlled Langmuir trough (KSV Minitrough, KSV Instruments, Helsinki, Finland), equipped with a platinum Wilhelmy plate was used to obtain the surface pressure-area (Π–A) isotherms of monolayers at the air/water interface. The temperature was maintained at 25 ± 0.1°C by an external water bath circulation. The volume of the trough was 80 ml. The cleanliness of the surface was ensured by closing the barriers, followed by aspiration of the subphase surface, before each experiment. Each experiment was started when the fluctuation of the surface pressure was <0.1 mN/m during the compression cycle. Lipid mixture (DOPC/DPPC (1:1)) and DOPC/DPPC mixture with ciprofloxacin or moxifloxacin at different molar proportions (1:1:0.1, 1:1:0.4, 1:1:1, and 1:1:2) were spread from a 1 mM (1:1:0.1, 1:1:0.4, and 1:1:1 molar ratios) or 2 mM (1:1:2) $CHCl_3/CH_3OH$ (2:1, v/v) solution on a Tris 10 mM subphase adjusted at pH 7.4. Thirty minutes were allowed for solvent evaporation from the interface. The air/water interface was then compressed with two Delrin barriers at a rate of 5.8 Å^2 molecule^{-1} min^{-1}. The reproducibility of the area values remained ~7%. The accuracy on surface pressure was within 0.1 mN/m.

Mean molecular area (A) of the components at the interface was calculated taking into account the percentage of fluoroquinolone remaining at the interface after 30 min. The following equation was used:

$$A = (A_{trough} \times 10^{16}/\mathrm{N})\,(MW \times 1000/C) \times V \times 10^3.$$

A_{trough} = Area of the trough (cm^2); N = Avogadro number (6.022×10^{23}); MW = Weighted average of the molecular weight of the components remaining at the interface; C = Weighted average of the concentration (mg/ml) of the components remaining at the interface; V = Sample volume spread at the interface.

Release of fluoroquinolones from lipid monolayer to aqueous phase—Langmuir experiments

The determination of the release of fluoroquinolones from lipid monolayer into the subphase (Tris buffer 10 mM, pH 7.4) was performed as described previously. In these experiments, mixed solutions of DOPC/DPPC/fluoroquinolone at different molar proportions (1:1:0.1, 1:1:0.4, 1:1:1, and 1:1:2) were spread from a $CHCl_3/CH_3OH$ (2:1, v/v) solution on the subphase until a surface pressure of 11.6 ± 0.8 mN/m was reached. Although this pressure is well below the estimated surface pressure of a biological membrane (31–34 mN/m (19,20)), it allows an accurate determination of the kinetics of the transfer of fluoroquinolones to the subphase. Immediately after spreading and every 5 min, two aliquots of 500 μl were taken from the subphase with a micropipette. Homogenization of the subphase was provided by a gentle constant stirring. Each experiment was replicated at least three times. The presence of lipids was detected by phospholipid assay and fluoroquinolones were assayed by fluorimetry.

Conformation and orientation of lipids in interaction with fluoroquinolones—ATR-FTIR spectroscopy

Attenuated total reflection Fourier transform infra-red (ATR-FTIR) is particularly well suited for the study of membranes and to characterize the effect of drug interacting with lipids on conformation and orientation of acyl chains of phospholipids (21). This technique is based on internal reflection of the infrared light within an internal reflection germanium plate, which creates an evanescent field at the surface of the plate where the lipid bilayer (and eventually the bound proteins or drugs) resides (22). After deposit of lipids on the germanium plate, while evaporating, capillary forces flatten the membranes which spontaneously form oriented multilayer arrangements (23). The internal reflection element was a 52 × 20 × 2 mm trapezoidal germanium ATR plate (ACM, Villiers St. Frédéric, France) with an aperture angle of 45° yielding 25 internal reflections.

Infra-red spectra were obtained on a model No. IFS55 FTIR spectrophotometer (Bruker, Ettlingen, Germany) purged with N_2 (as described previously (24)). Spectra were recorded with 2 cm^{-1} spectral resolution with a broad-band MCT detector provided by Bruker between 4000 and 800 cm^{-1}; 128 scan were averaged for one spectrum. A modified continuous flow ATR setup was equipped with a polarizer that can be oriented parallel or perpendicular to the incidence plane. Fifteen microliters of the sample containing DPPC with different lipid/antibiotic molar ratios (DPPC/drug ratio: 1:0, 1:0.2, 1:0.5, 1:1, and 1:2) were dried under a stream of nitrogen on one side of the germanium internal reflection element using an incident angle of 45° at 20°C. An elevator under computer control made it possible to move the

whole setup along a vertical axis (built for us by WOW Company, Nannine, Belgium). The software used for data processing was written under MatLab 6.5 (The MathWorks, Natick, MA). All spectra were corrected for water vapor contribution and CO_2 and finally apodized at a resolution of 4 cm^{-1}.

To analyze the conformation of lipids, nonpolarized spectra were recorded. The hydrocarbon chain in α-position of DPPC in the gel state is in all *trans* from the ester group down to the methyl group. This conformation allows a resonance to occur between the ester group and the CH_2 groups of the chain, giving rise to the so-called $\gamma_w(CH_2)$ progression between 1200 and 1350 cm^{-1} (peaks at 1200, 1221, 1246, 1266, 1286, 1309, and 1330 cm^{-1}). The proportion of the α-chains in the all-*trans* conformation was evaluated from the area of the band at 1200 cm^{-1} relative to the C-H stretching vibrations of the CH_2 and CH_3 at 3000–2800 cm^{-1}.

To get information about the orientation of lipids and chain ordering, dichroic spectra of DPPC and DPPC in interaction with ciprofloxacin or moxifloxacin were obtained by subtracting the spectrum measured with a perpendicular polarization of the incident light ($A_\perp$) from the spectrum of absorbance measured with a parallel polarization of the incident light ($A_{//}$). The angle between the molecular axis and the membrane normal was calculated as reviewed in Goormaghtigh et al. (21) using the STDWAVE program developed in the laboratory of one of the authors.

RESULTS

AFM imaging of DOPC/DPPC bilayers incubated with ciprofloxacin and moxifloxacin

To gain insight into ciprofloxacin- and moxifloxacin-membrane interactions, supported lipid bilayers made of DOPC/DPPC were prepared by fusion of unilamellar vesicles on mica. Time-lapse AFM topographic images were recorded in solution, in the absence and in the presence of fluoroquinolones.

In the absence of drug, classical images previously published (13) were obtained. They displayed two discrete height levels reflecting phase separation between gel phase DPPC and liquid-crystalline phase DOPC. The DPPC gel domains were well defined and homogenous, with a size ranging from 0.15 to 1.5 μm. The height difference between DPPC domains and the fluid DOPC matrix was 1.10 $\pm$ 0.05 nm.

When bilayers were incubated with either ciprofloxacin (*top panels*) or moxifloxacin (*bottom panels*), we observed a decrease of the size of DPPC domain with time (Fig. 2). The height differences between gel phase DPPC and fluid phase DOPC remains constant during the incubation. We note that for all incubation times the bilayer surface was devoid of defects, i.e., holes in the upper monolayer or in the bilayer were never observed. To evaluate the kinetics of the erosion process, a plot of the average domain areas was represented as a function of time. Fig. 3 shows that within a few hours, the area of DPPC domains decreased from 114.1 to 75.6 μm^2 and 83.3 to 42.3 μm^2 for ciprofloxacin and moxifloxacin, respectively. Thus, ciprofloxacin induced a decrease of the surface occupied by the DPPC domain of 27%. This erosion process was more marked with moxifloxacin since it reached a value of 58%. The kinetic trend was also different for ciprofloxacin compared to moxifloxacin, as reflected by the linear and the exponential-like processes, respectively. This suggests that, in contrast with ciprofloxacin, different regimes of erosion had to be distinguished with moxifloxacin. To assess whether such an alteration of DPPC domains could be due to mechanical perturbation by the scanning tip, we performed the same measurements on a control bilayer that was not incubated with drugs. We obtained an area decrease of 3% indicating that the time-dependent erosion of the DPPC gel domains is due to the action of the antibiotics rather than to a simple scanning effect.

Partition of ciprofloxacin and moxifloxacin measured by phase transfer

The differences in behavior of ciprofloxacin and moxifloxacin observed by AFM experiments might be related to their ability to partition between aqueous and hydrophobic environments. To this end, we followed the transfer of the ciprofloxacin and moxifloxacin from an aqueous to a lipidic phase, using egg yolk phosphatidylcholine dissolved in chloroform (Fig. 4). Mostly, egg yolk phosphatidylcholine is a mixture of C16 and C18 saturated alkyl chains at C-1, and C18 unsaturated alkyl chain at C-2. Its thickness and degree of hydration, as well as mean acyl-chain area, are well known (25,26). Egg yolk phosphatidylcholine is commonly used to mimic lipid membranes and shows close characteristics of synthetic lipids used in this study. For example, the thickness of egg yolk phosphatidylcholine bilayer was estimated to be 30 Å, a close value to the thickness of DPPC (29.3 Å) and DOPC (30 Å; deduced from the thickness of DSPC, which has the same carbon number in the alkyl chain as DOPC (26)). In the absence of lipid, more than 40% of ciprofloxacin was detected in the aqueous phase, while only 5% of moxifloxacine was found in these conditions. A huge amount of this latter was found at the interface. The addition of lipids to the organic phase, with a lipid/drug molar ratio up to 50:1 did not change significantly the drug phase transfer.

Transfer of fluoroquinolones from lipid monolayer to aqueous phase

To get more insight on the partition of fluoroquinolones between lipid and aqueous phases, we investigated the ability of fluoroquinolones to be released from a lipid monolayer to an aqueous phase, by using the Langmuir trough technique. The amount of fluoroquinolone found in the subphase increased with the initial quantity of fluoroquinolone in the monolayer (data not shown). For a DOPC/DPPC/fluoroquinolone ratio 1:1:2, a plateau value was reached within 10 min for ciprofloxacin and 15 min for moxifloxacin (Fig. 5). At this equilibrium state, the percentage of fluoroquinolone detected in the subphase was clearly higher for ciprofloxacin (~70%) as compared to moxifloxacin (at ~40%). In the experimental conditions used, no lipid was detected by phospholipid assay

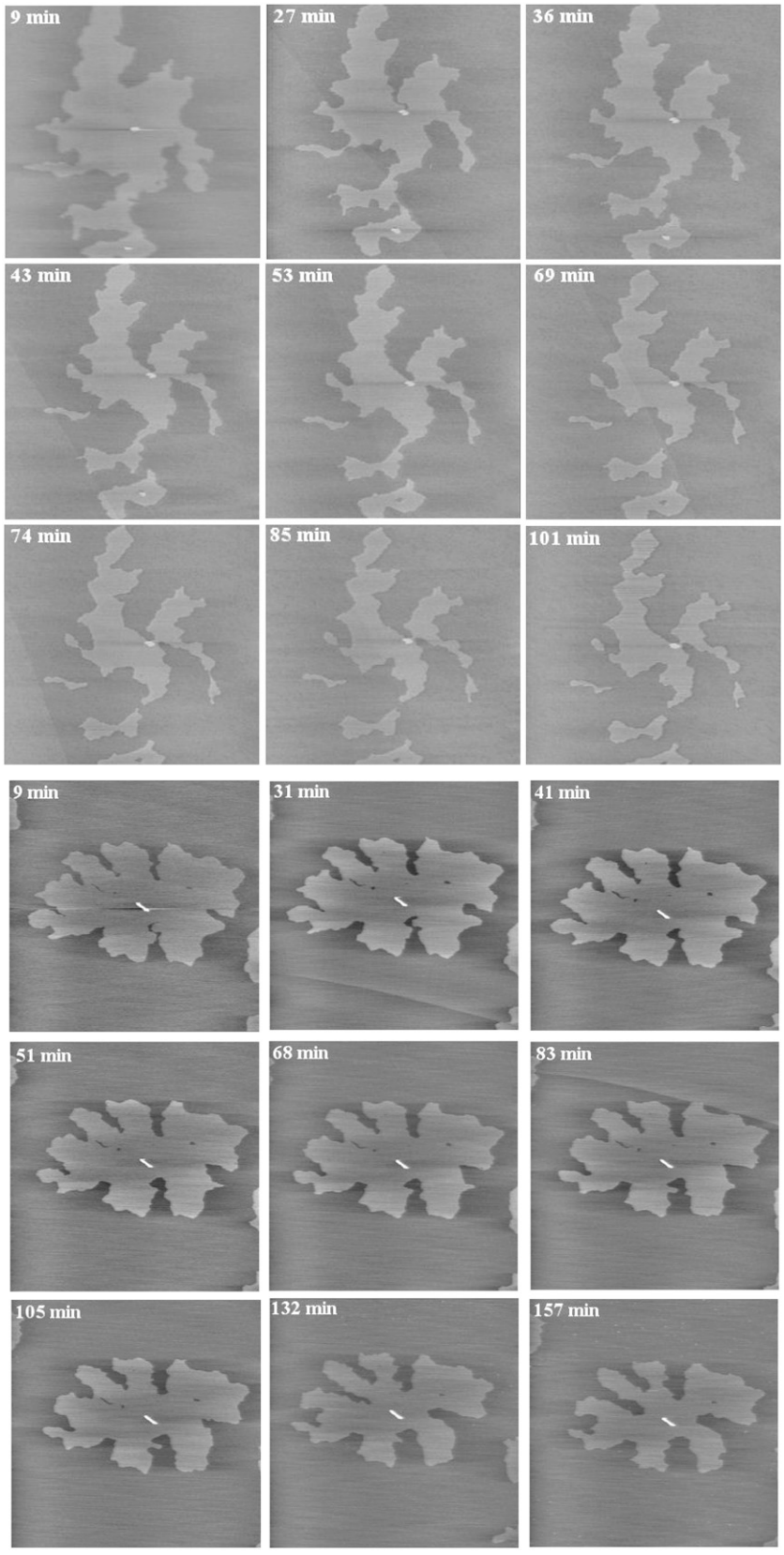

FIGURE 2 AFM height images (20 μm $\times$ 20 μm (*top panels*) or 15 μm $\times$ 15 μm (*bottom panels*), z-scale: 5 nm) of a mixed DOPC/DOPC (1:1, mol/mol) bilayer recorded in Tris 10 mM, NaCl 100 mM buffer, pH 7.4 containing 1 mM of ciprofloxacin (*top panels*) or moxifloxacin (*bottom panels*) at increasing incubation time.

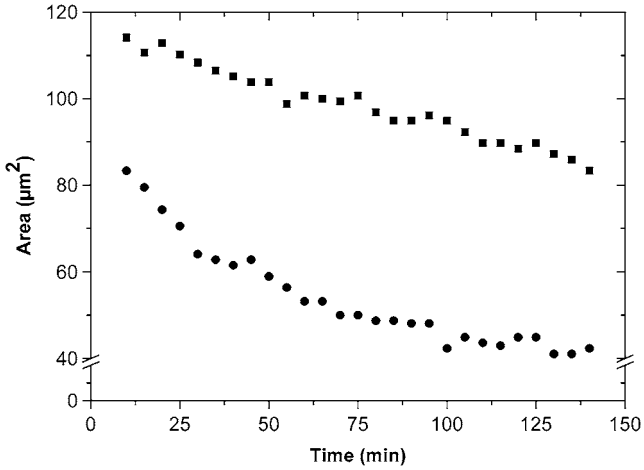

FIGURE 3 Evolution of the area of the DPPC domain (Fig. 2) with time for DOPC/DPPC bilayers incubated in Tris 10 mM, NaCl 100 mM buffer, pH 7.4, containing 1 mM of ciprofloxacin (*solid squares*) or moxifloxacin (*solid circles*).

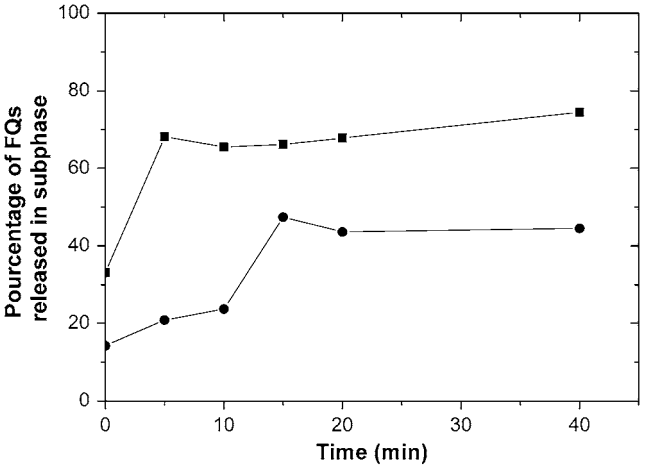

FIGURE 5 Kinetics of the release of fluoroquinolones from the mixed lipids/fluoroquinolones monolayer to the subphase (10 mM Tris pH 7.4, 25°C). Results are expressed as the percentage of fluoroquinolones initially present in the monolayer. Ciprofloxacin, ■; and moxifloxacin, ●. Molar proportion of DPPC/DOPC/fluoroquinolones (1:1:2).

in the buffer that supports the lipid-fluoroquinolone monolayer.

Conformational analysis of the interactions between fluoroquinolones and lipids

In an attempt to correlate our experimental data with molecular modeling, the interaction of ciprofloxacin and moxifloxacin with a model membrane was calculated using the IMPALA method. This procedure was used to study the membrane behavior of both molecules when crossing the bilayer from the hydrophilic environment to the hydrophobic.

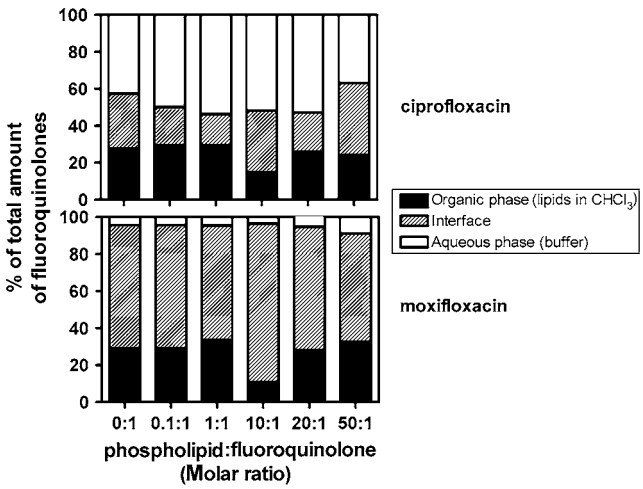

FIGURE 4 Phase transfer of ciprofloxacin (1 μM, *top panel*) and moxifloxacin (1 μM, *bottom panel*) in Tris buffer pH 7.4 against increasing amounts of PC (from 0.1:1 up to a lipid/drug ratio of 50:1) in chloroform. (*Open bars*) Drug in aqueous phase. (*Hatched bars*) Drug at interface (calculated from the difference between initial drug concentration and measured drug in aqueous and organic phase). (*Solid bars*) Drug in organic phase. Experiments were reproduced at least three times with similar results.

Fig. 6 *A* shows the most stable position of each molecule into the membrane. Both fluoroquinolones are clearly located at the hydrophilic-hydrophobic interface. The molecules were embedded into the membrane, with their mass center near the phospholipid headgroup/acyl-chain interface (~13 Å from the bilayer center), as shown on the plot of the mass center position versus the restraints (Fig. 6 *B*). It should be noted that differences were seen between ciprofloxacin and moxifloxacin. The interaction of moxifloxacin notably appeared more favorable than that of ciprofloxacin, since the restraint value of the most stable position was 1.5 kcal/mol lower for moxifloxacin as compared to ciprofloxacin.

Effect of fluoroquinolones on lipid monolayer—surface-pressure isotherms

To investigate the ability of fluoroquinolones to modify the surface pressure versus area isotherms of DOPC/DPPC (1:1) monolayers, we investigated the effect of increasing amounts of antibiotics on these isotherms curves.

For the sake of accuracy, we took into account the release of fluoroquinolones from the lipid to the aqueous phases in the determination of the quantity of drug remaining in the monolayer at the air-water interface, on the monolayers isotherms. We therefore recalculated the monolayer compression isotherms using the proportion of fluoroquinolones remaining in the monolayer after 30 min. Results are illustrated in Fig. 7.

The curve corresponding to pure DOPC/DPPC (1:1) is in perfect agreement with the one already reported (27). As already evoked by Montero et al. (28), pure ciprofloxacin or moxifloxacin does not form a film at the air-water interface.

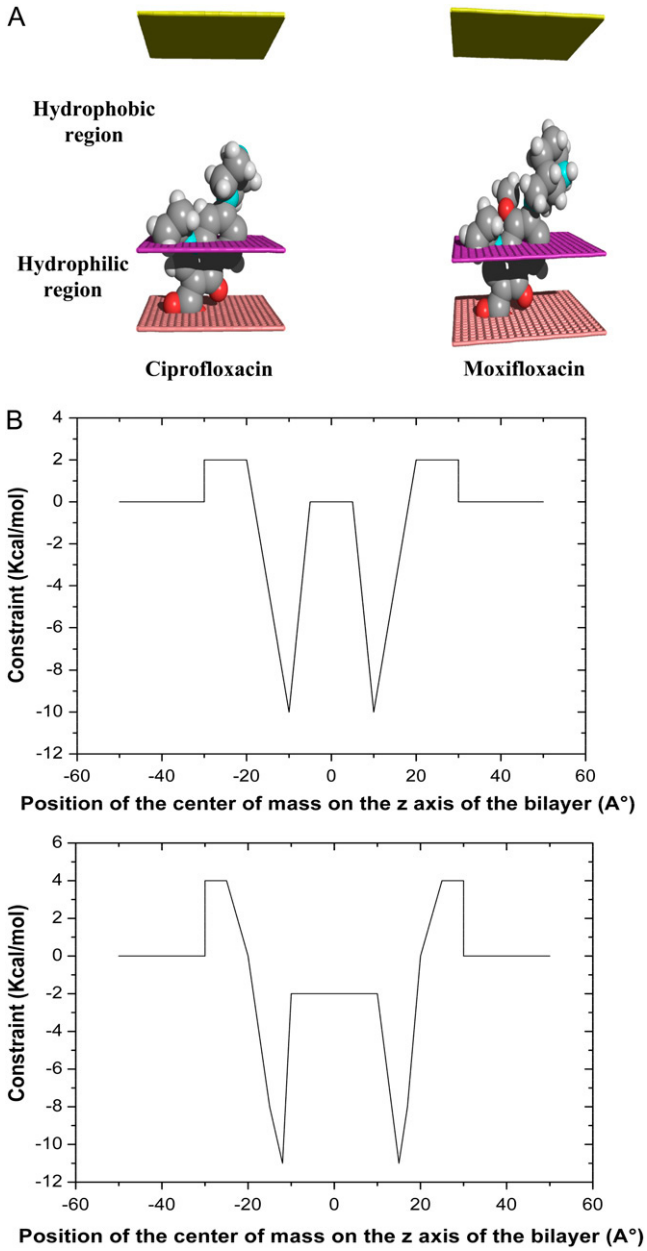

FIGURE 6 Molecular modeling of the interactions between ciprofloxacine and moxifloxacine with an implicit membrane. (*A*) Most favorable position of the ciprofloxacin (*left*) and moxifloxacin (*right*) in a lipid bilayer. (*B*) Restraints versus the position of the ciprofloxacin (*up*) and moxifloxacin (*down*) in the bilayer. Constraints are expressed in kcal/mol and the positions are expressed in Ångstroms.

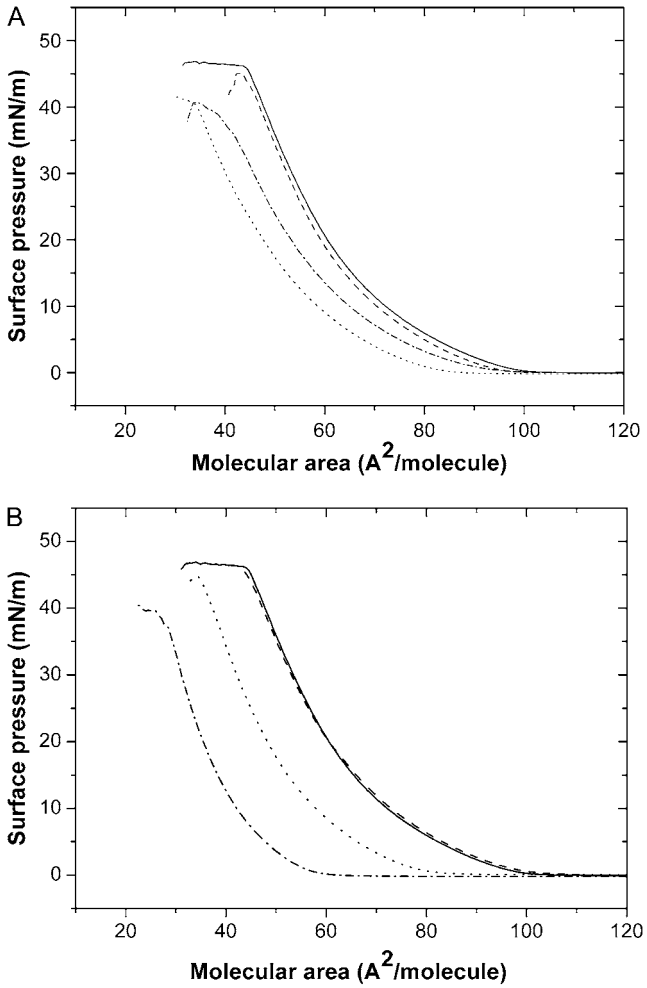

FIGURE 7 Surface pressure-molecular area isotherms of DOPC/DPPC in the presence of ciprofloxacin (*A*) and moxifloxacin (*B*), on a subphase of 10 mM Tris at pH 7.4 and 25°C. Mean molecular area were corrected to take into account the percentage of fluoroquinolone remaining at the interface. DOPC/DPPC/drug molar ratio were 1:1:0 (*continuous line*), 1:1:0.4 (*discontinuous line*), 1:1:1 (*dotted line*), and 1:1:2 (*dash-dotted line*).

In presence of fluoroquinolones, the isotherms were shifted toward the small molecular areas. This effect is more pronounced with moxifloxacin (Fig. 7 *B*) than ciprofloxacin (Fig. 7 *A*).

In addition, fluoroquinolones also affected the collapse pressure: 46.0 mN/m for DOPC/DPPC; 45.9 mN/m, 44.8 mN/m, and 37.7 mN/m, for initial proportions of DOPC/DPPC/moxifloxacin of 1:1:0.4, 1:1:1, and 1:1:2, respectively; and 46.0 mN/m, 40.5 mN/m, and 37.8 mN/m, for initial proportions of DOPC/DPPC/ciprofloxacin of 1:1:0.4, 1:1:1, and 1:1:2, respectively. This disruption of the lipid monolayer stability at a high compression level is more pronounced at a high level of fluoroquinolone in the mixed monolayer.

Effect of fluoroquinolones on lipid conformation—ATR-FTIR

Because the lower area occupied by lipids in presence of fluoroquinolones might be partly due to a change in the orientation of lipid at the interface by straightening up their fatty acid chains, we used ATR-FTIR to investigate the effect of ciprofloxacin and moxifloxacin on conformation and orientation of acyl chain of lipids.

Nonpolarized ATR-FTIR spectra of supported layers of DPPC, drug (ciprofloxacin or moxifloxacin), and DPPC/drug at a molar ratio of 1:1 were recorded (Fig. 8, *top panel*). As the drug proportion increased, the drug spectrum appeared in the DPPC/drug mixture spectrum, notably at 1630 cm^{-1}. Interestingly, in DPPC/drug spectra, the DPPC ν(C=O) band at 1736 cm^{-1} was modified in terms of frequency and shape, suggesting a modification of the interfacial lipid carbonyl groups. Analysis of the lipid C-H wagging ($\nu_w(CH_2)$) allowed us to get information on lipid chain conformation and proportion of the chains in the all-*trans* conformation (23). Here the wagging band at 1200 cm^{-1} was selected because it has little overlap with other lipid or drug absorption. As shown in Fig. 8, *bottom panel*, area evolution of DPPC peak at 1206–1193 cm^{-1} as function of increasing amounts of fluoroquinolones decreased by up to 60 and 72% for ciprofloxacin and moxifloxacin, respectively. These data indicated a loss of all-*trans* conformation and the appearance of a kink somewhere between C-2 and C-6 of the chain.

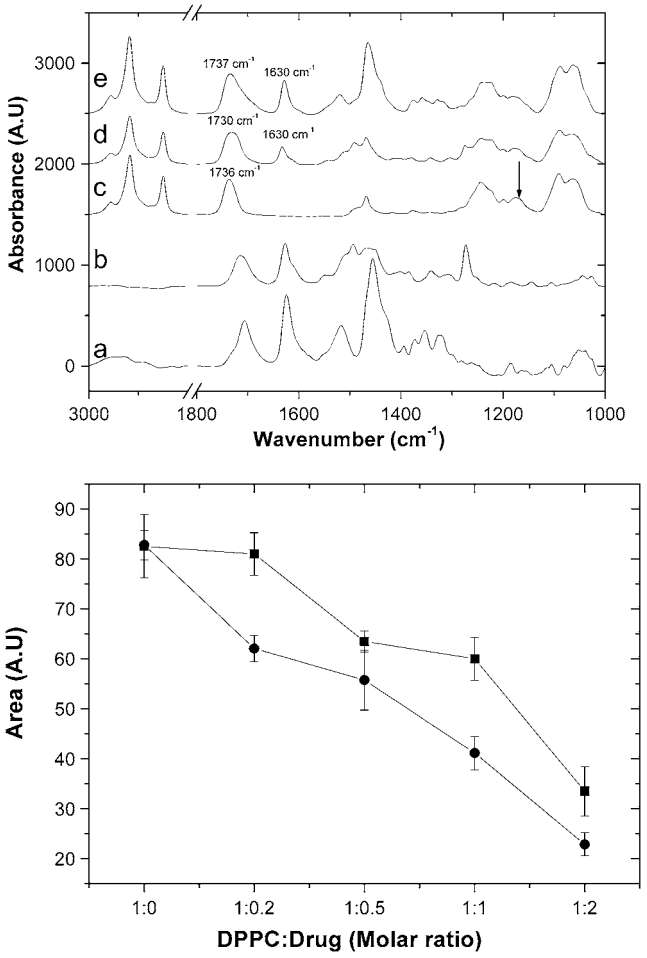

FIGURE 8 Fluoroquinolones effect on the conformation of DPPC monolayer as revealed by the infrared absorbance spectra. (*Top panel*) ATR-FTIR spectra of moxifloxacin (*a*), ciprofloxacin (*b*), DPPC (*c*), DPPC/ciprofloxacin (*d*), and DPPC/moxifloxacin (*e*). DPPC was used at 50 mg/ml and the molar ratio of lipid/drug was 1:1. (*Bottom panel*) Evolution of the peak area at 1200 cm^{-1} (wagging $\gamma_w(CH_2)$ band; integrated between 1206 and 1193 cm^{-1}) as a function of increasing lipid/drug ratio: 1:0, 1:0,2, 1:0,5, 1:1, and 1:2. Ciprofloxacin, ■; and moxifloxacin, ●.

Effect of fluoroquinolones on lipid orientation—ATR-FTIR

To get information on molecular orientation in the absence or in the presence of both fluoroquinolones, we took advantage from the fact that, in an ordered membrane deposited on the germanium crystal (oriented multilayers), all the molecules have the same orientation with respect to a normal to the germanium plate. Measuring the spectral intensity with two orientations of the incident-light electric field obtained with a polarizer allowed us to obtain information on several chemical groups of the lipid molecule. The dichroic spectrum of pure DPPC and DPPC/drug (molar ratio 1:1) mixture were obtained by subtracting the spectrum recorded with perpendicular-polarized light from that recorded with the parallel-polarized light using the lipid ν(C=O) band at 1780–1700 cm^{-1} as a reference (Fig. 9) (29). Interestingly the dichroic spectra of DPPC/drug mixture displayed strong dichroism for bands assigned to the drug, notably at 1630 and 1465 cm^{-1}, suggesting a well-organized, well-defined orientation of the drug in the DPPC bilayer. The orientation of the lipid acyl chain can be estimated from the wagging band ($\nu_w(CH_2)$). The dipole of this transition is oriented parallel to the all-*trans* chain (23). In turn, positive deviations of the dichroism spectrum demonstrate that the chains are mainly perpendicular to the germanium surface, i.e., perpendicular to the membrane plane since AFM recording demonstrated that membranes orient themselves parallel to the germanium surface, even when natural membranes are used (30). In Fig. 9, *bottom panel*, we plotted the area evolution of the wagging peak integrated between 1206 and 1193 cm^{-1} as a function of the DPPC/drug molar ratio. Both fluoroquinolones induced a marked and similar decrease of the area when they were added at low concentration (1:0.2 molar ratio). When the amounts of fluoroquinolones were increased, the area decreased further in presence of ciprofloxacin but remained almost stable for moxifloxacin. This observation was similar for the four wagging peaks (indicated by *arrows*; Fig. 9, *top panel*) (1275–1261 cm^{-1}, 1253–1240 cm^{-1}, 1229–1216 cm^{-1}, and 1206–1193 cm^{-1}).

To quantify the orientation of DPPC all-*trans* chains, we measured the dichroic ratio for wagging band at 1200 cm^{-1}. R^{ATR} ($A_{//}/A_{\perp}$) was 6.8 with an isotropic dichroic ratio of 1.33 (calculated from ν(C=O) band at 1755–1750 cm^{-1} (29)). On the basis of this determination, the angle between the acyl chains of DPPC and the normal at the germanium surface was found to be 21°. The same calculation was done in the presence of fluoroquinolones at a lipid/drug ratio of 1:1. The angle was 27° and 20° in the presence of ciprofloxacin and moxifloxacin, respectively. These data suggested that in contrast to ciprofloxacin, moxifloxacin had no effect on the

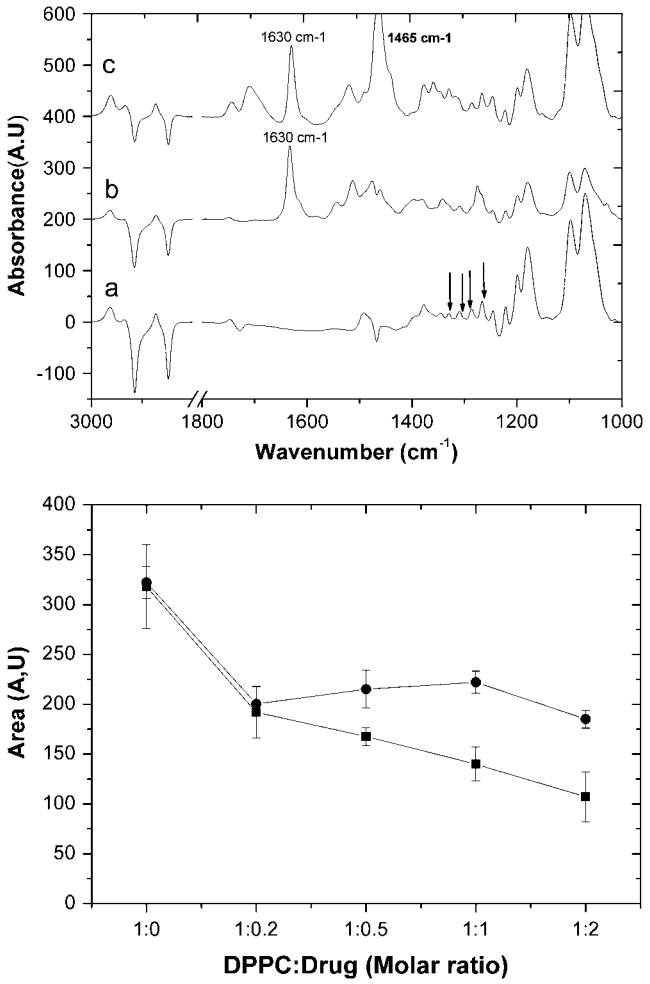

FIGURE 9 Fluoroquinolones effect on the orientation of DPPC monolayer as revealed by ATR-FTIR dichroic spectra. (*Top panel*) Polarized ATR-FTIR spectra of DPPC (*a*), DPPC/ciprofloxacin (*b*), and DPPC/moxifloxacin (*c*). DPPC was used at 50 mg/ml and the molar ratio of lipid/drug was 1:1. Wagging $\gamma_w(CH_2)$ bands are indicated by arrows. (*Bottom panel*) Area evolution of dichroic peak of 1200 cm^{-1} of DPPC in the presence of fluoroquinolones. Integration area of dichroic $\gamma_w(CH_2)$ band (integrated between 1206 and 1193 cm^{-1}) was plotted versus DPPC/drug molar ratio as indicated, in the presence of ciprofloxacin, ■; and moxifloxacin, ●.

orientation of the acyl chains and did not induce additional disorder.

DISCUSSION

Our previous studies showed differences in accumulation and intracellular activity between ciprofloxacin and moxifloxacin that may actually result from their differential susceptibility to efflux by the ciprofloxacin transporter (1). Moxifloxacin differs from ciprofloxacin by the presence of a C-8 methoxy group and a bulkier C-7 (octahydropyrrolo (3.4)-pyridinyl versus piperazinyl for ciprofloxacin) substituent (31). As previously shown (32), these changes resulted in an increase in hydrophobicity (log $D \sim -0.28$(33) for moxifloxacin and -0.79(34) for ciprofloxacin). However, these structural modifications between moxifloxacin and ciprofloxacin may not account for the differences in the pK values (from 6.25 to 6.09 (33,36), since these differences are below the experimental errors. Interestingly, these compounds were much weaker acids than aromatic carboxylic acids. The reduced acidity may be ascribed to the formation of an intramolecular hydrogen bond between the carboxyl and neighboring keto groups in the quinoline ring, resulting in stabilization of the protonated form of the carboxyl group.

The activity of efflux pumps in general, and those involved for ciprofloxacin or moxifloxacin cellular accumulation, in particular, is closely related to the interaction of the drug with lipids, regardless of the model proposed for their activity (i.e., flippase or hydrophobic vacuum cleaner). We therefore looked at the influence of the small changes in chemical structure between moxifloxacin and ciprofloxacin on their interaction with lipids. To address this crucial question, the interactions between model lipid membranes and the two fluoroquinolones were probed using a variety of techniques.

Nanoscale investigations by AFM revealed different behaviors for ciprofloxacin and moxifloxacin. AFM imaging showed a reduction of the size of the DPPC gel phase domains in presence of fluoroquinolones. The erosion process is greater for moxifloxacin compared to ciprofloxacin, and follows an exponential-like function-decrease for moxifloxacin and a linear-decrease for ciprofloxacin. Considering both molecules, the difference in their ability to erode the DPPC gel phase domains might be due to a better insertion of the moxifloxacin than the ciprofloxacin into the fluid matrix, and a more marked decrease of the line tension at the boundary of the DOPC/DPPC phases resulting from a fluidification of the DPPC.

This marked effect of moxifloxacin on lipids as compared to ciprofloxacin was confirmed by conformational analysis. In accordance with experimental data obtained for ciprofloxacin (37) or grepafloxacin (38), the fluoroquinolones are located at the lipid-water interface, near the first carbons of the acyl chains. Both molecules showed a minimum of energy when they are at the phospholipids headgroup/acyl-chains interface, and the interaction energy rose markedly when the molecule was forced into the hydrophobic domain. This energy increase was less marked for moxifloxacin as compared to ciprofloxacin, suggesting a higher affinity of moxifloxacin for lipid phase.

Taken together, our data suggest that ciprofloxacin and moxifloxacin interact in a very different way with lipids. The major challenge, however, is to understand the mechanism, at a molecular level, unraveling the interaction between lipids and fluoroquinolones and the path of these antibiotic molecules through lipid layers. Previous data reported by Montero et al. (28) showed a shift of the surface pressure-area isotherms of monolayer toward a lower area per molecule in the presence of ciprofloxacin. We extended these data to one major fluoroquinolone used in clinics, moxifloxacin. To go further in the mechanism involved, we monitored the amount

of fluoroquinolones in the subphase after the monolayer compression. In agreement with the hypothesis based on a dissolving effect in the subphase (39), we did find fluoroquinolones therein with a higher proportion of ciprofloxacin as compared to moxifloxacin. Taking into account the amount of fluoroquinolones present inside the monolayer, we corrected the surface-pressure-area isotherms of the monolayer and again observed a shift toward a lower area per molecule in the presence of fluoroquinolones. This effect was more marked with moxifloxacin as compared to ciprofloxacin. The dissolving effect in the aqueous phase is therefore probably essential, although not fully sufficient, to explain the condensing effect of fluoroquinolones.

Thus, we investigated a change in the lipid chain conformation and orientation using ATR-FTIR technique (21,40). Indeed, the drug-induced area condensation of lipids can derive from the acyl ordering attained when *trans-gauche* isomerization about the carbon-carbon bonds is reduced. The *trans* conformation is the most stable and has an estimated energy barrier of 3.5 kcal/mol to rotate past the eclipsed configuration to the *gauche* form. The all-*trans* conformation allows the chain to be maximally extended, whereas a *gauche* bond alters the direction of the chain inducing a kink in the chain. Our results clearly indicated that moxifloxacin has a higher ability than ciprofloxacin to markedly decrease the number of all-*trans* conformation. The related change in the packing of the acyl chains might allow moxifloxacin to be located in the pocket created by the presence of a kink in the acyl chain. In contrast, with ciprofloxacin, the appearance of a kink from the all-*trans* chain conformation would be less marked, suggesting a less important change in lipid packing. Interestingly, both condensing effects (lower area of mixed monolayer lipids/fluoroquinolones) have also been described when cholesterol was added to fluid-phase phosphatidylcholine (41–43).

The disorder in the lipid chains revealed by the decrease of all-*trans* conformations has also been analyzed in terms of orientation and tilt between the molecular axis (the membrane normal) and the transition dipole moments. In this analysis, the ciprofloxacin showed an additional cause of disorder, because it modifies the orientation of the acyl chain in relation to its higher ability to be released in an aqueous phase after monolayer compression.

Differences in the charge distribution of the molecule at the physiological pH could also explain changes for drug membrane location and bound hydration shell surrounding the headgroup of membrane lipids, which, in turn, could partly explain the more condensing effect of moxifloxacin as compared to ciprofloxacin. Moreover, the determinations were made in aqueous environment, whereas the condensing effect of moxifloxacin involved the presence of the drug with a lipidic phase.

All together, we showed that the condensing effect of fluoroquinolones on lipid layer resulted not only from a dissolving mechanism but also from an alteration of the intramolecular acyl-chain order in relation to a reduction in *trans-gauche* isomerization about the carbon-carbon bonds, and change in the average molecular tilt of lipid acyl chain of DPPC. The two fluoroquinolones investigated showed difference in their effects. Ciprofloxacin had a lower ability to decrease the all-*trans* conformation of lipid chains than moxifloxacin but showed a higher capacity to affect the orientation of lipid chains and to disorder the membrane. These effects might explain its higher ability to be released from the lipid monolayer to aqueous phase and its lower effect on surface pressure-area isotherms of monolayers. In contrast, moxifloxacin has a lower capacity to induce membrane disorder and does not change the tilt between the molecular axis and the transition dipole moment. Moxifloxacin has also a higher tendency to decrease the number of all-*trans* conformations with increase of kink, creating a pocket in which moxifloxacin can be located. This can explain why the amount of moxifloxacin in the aqueous phase was lower than that found for ciprofloxacin and why the mean molecular area of lipids/fluoroquinolones monolayers after compression is significantly lower in the presence of moxifloxacin as compared to ciprofloxacin.

This model is entirely compatible with the physicochemical characteristics of the two fluoroquinolones. It suggested that small structural differences among fluoroquinolones (notably overall molecular hydrophobicity (P_{app} = 0.089 vs. 0.031 for ciprofloxacin and moxifloxacin, respectively (45)), bulkiness, and/or the internal dynamics of the C-7 substituent, could be important for drug lipid interactions and lipid packing. The diazabicyclonyl-ring at position 7 of moxifloxacin, by aligning the *sn*-2 chain, probably contributes to the higher tendency of this antibiotic to induce a decrease of all-*trans* configuration as compared to ciprofloxacin. This is in line with data reported with *n*-alkyl-piperazinyl-ciprofloxacin (39).

In conclusion, we provided a comprehensive picture of the interaction of the two major fluoroquinolones ciprofloxacine and moxifloxacine with lipids, and elucidate fundamental issues such as the relationship between lipid chain conformation and orientation with changes in membrane properties as determined by Langmuir studies and the ability of drugs to diffuse through membranes. All these parameters might be related to the activity of membranous proteins. Our work notably showed that an increase in drug lipophilicity and addition of a bulky moiety (moxifloxacin versus ciprofloxacin) produced marked changes in the packing of lipids. This was concomitant with a lower release of the more lipophilic drug from lipid monolayer and with a potential inefficient activity of efflux proteins which could be involved in a kind of futile cycle resulting in an increase in cellular accumulation (1). So far, progress in understanding the structure-function relationships of membranes and understanding of the lipid-drug interaction appears to be of crucial importance in understanding the mechanisms involved in cellular drug accumulation.

We thank Professor J. Poupaert for fruitful discussions, and Professors F. Van Bambeke and P. M. Tulkens for supporting the project.

R.B., E.G., L.L., M.D., and Y.D. are, respectively, Research Directors and Research Associates of the National Foundation for Scientific Research. H.B. is an assistant and doctoral fellow of the Université Catholique de Louvain. The support of the Région Wallonne, of the National Foundation for Scientific Research, of the Université Catholique de Louvain (Fonds Spéciaux de Recherche, Actions de Recherche Concertées), and of the Federal Office for Scientific, Technical and Cultural Affairs (Interuniversity Poles of Attraction Program), is gratefully acknowledged. We also thank Bayer Healthcare AG for providing us fluoroquinolone antibiotics.

REFERENCES

1. Michot, J. M., C. Seral, F. Van Bambeke, M. P. Mingeot-Leclercq, and P. Tulkens. 2005. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. *Antimicrob. Agents Chemother.* 49:2429–2437.
2. Bakker-Woudenberg, I. A., M. T. ten Kate, L. Guo, P. Working, and J. W. Mouton. 2001. Improved efficacy of ciprofloxacin administered in polyethylene glycol-coated liposomes for treatment of *Klebsiella pneumoniae* pneumonia in rats. *Antimicrob. Agents Chemother.* 45:1487–1492.
3. Bakker-Woudenberg, I. A., M. T. ten Kate, L. Guo, P. Working, and J. W. Mouton. 2002. Ciprofloxacin in polyethylene glycol-coated liposomes: efficacy in rat models of acute or chronic *Pseudomonas aeruginosa* infection. *Antimicrob. Agents Chemother.* 46:2575–2581.
4. Wong, J. P., H. Yang, K. L. Blasetti, G. Schnell, J. Conley, and L. N. Schofield. 2003. Liposome delivery of ciprofloxacin against intracellular *Francisella tularensis* infection. *J. Control. Release.* 92:265–273.
5. Rolston, K. V., D. Yadegarynia, D. P. Kontoyiannis, I. I. Raad, and D. H. Ho. 2006. The spectrum of Gram-positive bloodstream infections in patients with hematologic malignancies, and the in vitro activity of various quinolones against Gram-positive bacteria isolated from cancer patients. *Int. J. Infect. Dis.* 10:223–230.
6. Bounds, S. J., R. Nakkula, and J. D. Walters. 2000. Fluoroquinolone transport by human monocytes: characterization and comparison to other cells of myeloid lineage. *Antimicrob. Agents Chemother.* 44:2609–2614.
7. Hirota, M., T. Totsu, F. Adachi, K. Kamikawa, J. Watanabe, S. Kanegasaki, and K. Nakata. 2001. Comparison of antimycobacterial activity of grepafloxacin against *Mycobacterium avium* with that of levofloxacin: accumulation of grepafloxacin in human macrophages. *J. Infect. Chemother.* 7:16–21.
8. Hara, T., H. Takemura, K. Kanemitsu, H. Yamamoto, and J. Shimada. 2000. Comparative uptake of grepafloxacin and ciprofloxacin by a human monocytic cell line, THP-1. *J. Infect. Chemother.* 6:162–167.
9. Seral, C., M. Barcia-Macay, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke. 2005. Comparative activity of quinolones (ciprofloxacin, levofloxacin, moxifloxacin and garenoxacin) against extracellular and intracellular infection by *Listeria monocytogenes* and *Staphylococcus aureus* in J774 macrophages. *J. Antimicrob. Chemother.* 55:511–517.
10. Fresta, M., S. Guccione, A. R. Beccari, P. M. Furneri, and G. Puglisi. 2002. Combining molecular modeling with experimental methodologies: mechanism of membrane permeation and accumulation of ofloxacin. *Bioorg. Med. Chem.* 10:3871–3889.
11. Michot, J. M., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens. 2004. Active efflux of ciprofloxacin from J774 macrophages through an MRP-like transporter. *Antimicrob. Agents Chemother.* 48: 2673–2682.
12. Fernandez-Teruel, C., I. Gonzalez-Alvarez, V. G. Casabo, A. Ruiz-Garcia, and M. Bermejo. 2005. Kinetic modeling of the intestinal transport of sarafloxacin. Studies in situ in rat and in vitro in Caco-2 cells. *J. Drug Target.* 13:199–212.
13. Berquand, A., N. Fa, Y. F. Dufrene, and M. P. Mingeot-Leclercq. 2005. Interaction of the macrolide antibiotic azithromycin with lipid bilayers: effect on membrane organization, fluidity, and permeability. *Pharm. Res.* 22:465–475.
14. Siarheyeva, A., J. J. Lopez, and C. Glaubitz. 2006. Localization of multidrug transporter substrates within model membranes. *Biochemistry.* 45:6203–6211.
15. Hinrichs, J. W., K. Klappe, I. Hummel, and J. W. Kok. 2004. ATP-binding cassette transporters are enriched in non-caveolar detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs) in human multidrug-resistant cancer cells. *J. Biol. Chem.* 279:5734–5738.
16. Hinrichs, J. W., K. Klappe, and J. W. Kok. 2005. Rafts as missing link between multidrug resistance and sphingolipid metabolism. *J. Membr. Biol.* 203:57–64.
17. Laurent, G., M. B. Carlier, B. Rollman, F. Van Hoof, and P. M. Tulkens. 1982. Mechanism of aminoglycoside-induced lysosomal phospholipidosis: in vitro and in vivo studies with gentamicin and amikacin. *Biochem. Pharmacol.* 31:3861–3870.
18. Ducarme, P., M. Rahman, and R. Brasseur. 1998. IMPALA: a simple restraint field to simulate the biological membrane in molecular structure studies. *Proteins.* 30:357–371.
19. Demel, R. A., W. S. Geurts van Kessel, R. F. Zwaal, B. Roelofsen, and L. L. van Deenen. 1975. Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers. *Biochim. Biophys. Acta.* 406:97–107.
20. Marsh, D. 1996. Lateral pressure in membranes. *Biochim. Biophys. Acta.* 1286:183–223.
21. Goormaghtigh, E., V. Raussens, and J. M. Ruysschaert. 1999. Attenuated total reflection infrared spectroscopy of proteins and lipids in biological membranes. *Biochim. Biophys. Acta.* 1422:105–185.
22. Tatulian, S. A. 2003. Attenuated total reflection Fourier transform infrared spectroscopy: a method of choice for studying membrane proteins and lipids. *Biochemistry.* 42:11898–11907.
23. Fringeli, U. P., and H. H. Gunthard. 1981. Infrared membrane spectroscopy. *Mol. Biol. Biochem. Biophys.* 31:270–332.
24. Fa, N., S. Ronkart, A. Schanck, M. Deleu, A. Gaigneaux, E. Goormaghtigh, and M. P. Mingeot-Leclercq. 2006. Effect of the antibiotic azithromycin on thermotropic behavior of DOPC or DPPC bilayers. *Chem. Phys. Lipids.* 144:108–116.
25. Komatsu, H., H. Saito, S. Okada, M. Tanaka, M. Egashira, and T. Handa. 2001. Effects of the acyl chain composition of phosphatidylcholines on the stability of freeze-dried small liposomes in the presence of maltose. *Chem. Phys. Lipids.* 113:29–39.
26. Hara, M., H. Yuan, Q. Yang, T. Hoshino, A. Yokoyama, and J. Miyake. 1999. Stabilization of liposomal membranes by thermozeaxanthins: carotenoid-glucoside esters. *Biochim. Biophys. Acta.* 1461:147–154.
27. Vie, V., N. Van Mau, E. Lesniewska, J. P. Goudonnet, F. Heitz, and C. Le Grimellec. 1998. Distribution of ganglioside G(M1) between two-component, two-phase phosphatidylcholine monolayers. *Langmuir.* 14:4574–4583.
28. Montero, M. T., J. Hernandez-Borrell, and K. M. W. Keough. 1998. Fluoroquinolone-biomembrane interactions: monolayer and calorimetric studies. *Langmuir.* 14:2451–2454.
29. Bechinger, B., J. M. Ruysschaert, and E. Goormaghtigh. 1999. Membrane helix orientation from linear dichroism of infrared attenuated total reflection spectra. *Biophys. J.* 76:552–563.
30. Ivanov, D., N. Dubreuil, V. Raussens, J. M. Ruysschaert, and E. Goormaghtigh. 2004. Evaluation of the ordering of membranes in multilayer stacks built on an ATR-FTIR germanium crystal with atomic force microscopy: the case of the H^+,K^+-ATPase-containing gastric tubulovesicle membranes. *Biophys. J.* 87:1307–1315.
31. Dalhoff, A., U. Petersen, and R. Endermann. 1996. In vitro activity of BAY 12–8039, a new 8-methoxyquinolone. *Chemotherapy.* 42:410–425.
32. Klopman, G., O. T. Macina, M. E. Levinson, and H. S. Rosenkranz. 1987. Computer automated structure evaluation of quinolone antibacterial agents. *Antimicrob. Agents Chemother.* 31:1831–1840.

33. Langlois, M. H., M. Montagut, J. P. Dubost, J. Grellet, and M. C. Saux. 2005. Protonation equilibrium and lipophilicity of moxifloxacin. *J. Pharm. Biomed. Anal.* 37:389–393.

34. Sun, J., S. Sakai, Y. Tauchi, Y. Deguchi, J. Chen, R. Zhang, and K. Morimoto. 2002. Determination of lipophilicity of two quinolone antibacterials, ciprofloxacin and grepafloxacin, in the protonation equilibrium. *Eur. J. Pharm. Biopharm.* 54:51–58.

35. Neves, P., A. Leite, M. Rangel, B. de Castro, and P. Gameiro. 2007. Influence of structural factors on the enhanced activity of moxifloxacin: a fluorescence and EPR spectroscopic study. *Anal. Bioanal. Chem.* 387:1543–1552.

36. Furet, Y. X., J. Deshusses, and J. C. Pechere. 1992. Transport of pefloxacin across the bacterial cytoplasmic membrane in quinolone-susceptible *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 36:2506–2511.

37. Hernandez-Borrell, J., and M. T. Montero. 2003. Does ciprofloxacin interact with neutral bilayers? An aspect related to its antimicrobial activity. *Int. J. Pharm.* 252:149–157.

38. Rodrigues, C., P. Gameiro, S. Reis, J. L. F. Lima, and B. de Castro. 2002. Interaction of grepafloxacin with large unilamellar liposomes: partition and fluorescence studies reveal the importance of charge interactions. *Langmuir.* 18:10231–10236.

39. Vazquez, J. L., M. T. Montero, S. Merino, O. Domenech, M. Berlanga, M. Vinas, and J. Hernandez-Borrell. 2001. Location and nature of the surface membrane binding site of ciprofloxacin: a fluorescence study. *Langmuir.* 17:1009–1014.

40. Pare, C., M. Lafleur, F. Liu, R. N. Lewis, and R. N. McElhaney. 2001. Differential scanning calorimetry and ^{2}H nuclear magnetic resonance and Fourier transform infrared spectroscopy studies of the effects of transmembrane α-helical peptides on the organization of phosphatidylcholine bilayers. *Biochim. Biophys. Acta.* 1511:60–73.

41. Smaby, J. M., M. Momsen, V. S. Kulkarni, and R. E. Brown. 1996. Cholesterol-induced interfacial area condensations of galactosylceramides and sphingomyelins with identical acyl chains. *Biochemistry.* 35:5696–5704.

42. Smaby, J. M., M. M. Momsen, H. L. Brockman, and R. E. Brown. 1997. Phosphatidylcholine acyl unsaturation modulates the decrease in interfacial elasticity induced by cholesterol. *Biophys. J.* 73:1492–1505.

43. Bin, X., S. L. Horswell, and J. Lipkowski. 2005. Electrochemical and PM-IRRAS studies of the effect of cholesterol on the structure of a DMPC bilayer supported at an Au_{111} electrode surface, part 1: properties of the acyl chains. *Biophys. J.* 89:592–604.

44. Sun, J., S. Sakai, Y. Tauchi, Y. Deguchi, G. Cheng, J. Chen, and K. Morimoto. 2003. Protonation equilibrium and lipophilicity of olamufloxacin (HSR-903), a newly synthesized fluoroquinolone antibacterial. *Eur. J. Pharm. Biopharm.* 56:223–229.

45. Piddock, L. J., and Y. F. Jin. 1999. Antimicrobial activity and accumulation of moxifloxacin in quinolone-susceptible bacteria. *J. Antimicrob. Chemother.* 43:Suppl B:39–42.