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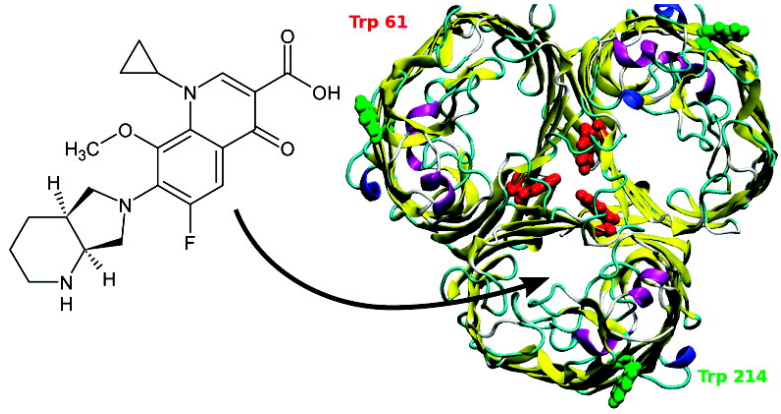

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Facilitated Permeation of Antibiotics across Membrane Channels − Interaction of the Quinolone Moxifloxacin with the OmpF Channel

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Abstract: The facilitated influx of moxifloxacin through the most abundant channel in the outer cell wall of Gram-negative bacteria was investigated. Molecular modeling provided atomic details of the interaction with the channel surface, revealed the preferred orientation of the antibiotic along its pathway, and gave an estimated time necessary for translocation. High-resolution conductance measurements on single OmpF trimers allowed the passages of individual moxifloxacin molecules to be counted. The average mean residence time of 50 μs is in agreement with the predicted strong interaction from the modeling. In contrast, control measurements with nalidixic acid, a hydrophobic antibiotic that rather permeates across the lipid membrane, revealed a negligible interaction. The spectral overlap of tryptophan with moxifloxacin was suitable for a FRET study of the protein−antibiotic interaction. Combining molecular dynamics simulations with selective quenching identified an interaction of moxifloxacin with Trp61 inside the OmpF channel, whereas nalidixic acid showed preferential interaction with Trp214 on the channel exterior. An understanding of the detailed molecular interactions between the antibiotic and its preferred channel may be used to develop new antibiotics with improved uptake kinetics.

Introduction

Infectious diseases caused by bacterial agents are a resurgent problem:[1,2] whereas the few new antimicrobial compounds are mainly modifications of existing classes, bacteria have developed several mechanisms to resist antibiotics, in some cases more than one acting concurrently.[3] Quinolones are among the most successful and widely used classes of antibiotics at present.[4−6] Their development over the years has fostered a wide range of applications: from narrow-spectrum antimicrobial agents used against Gram-negative bacteria (first-generation quinolones, such as nalidixic acid), to the most recent derivatives (fourth-generation agents, such as moxifloxacin) developed with a broader spectrum and enhanced activity against Gram-positive bacterial species, including *Streptococcus pneumoniae*, shifting the target site from the inhibition of topoisomerase IV to DNA gyrase.[7,8]

A first approach to counteracting bacterial resistance to these antibiotics involves a better understanding of the molecular basis governing their penetration across the outer cell wall via porins.[9−11] Recent studies have concluded that general diffusion through the porin OmpF (Figure 1, panels A and B) in Gram-negative bacteria plays an important role in the uptake of some antibiotics and that this uptake can often be considered the limiting step in their functionality.[12−15] However, the exact molecular mechanism of this uptake is not known,[16] and it is

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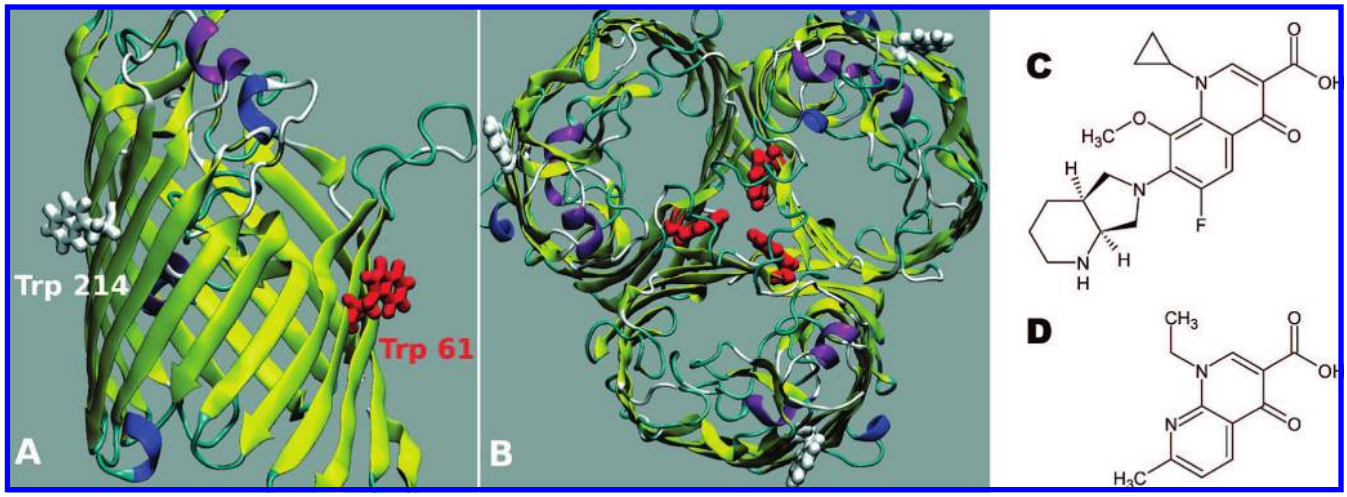

Figure 1. A and B: The structure of the OmpF trimer. The positions of the two Trp residues (Trp61 and Trp214) in each monomer are shown in red and white, respectively. Views of OmpF organization: (A) a monomer shown from the side and (B) the functional trimer shown from top. C: Chemical structure of moxifloxacin, D: chemical structure of nalidixic acid.

still debated whether the process takes place through the channel, through the lipid−channel interface, or through a direct lipid-mediated pathway within the phospholipid bilayer.[13,17]

For quinolones and β-lactams, two of the main classes of antibiotics, the resistant strains of pathogenic Gram-negative bacteria frequently have a deficiency in the expression of the general diffusion porin OmpF, and alterations of porins or production of porins exhibiting narrow channels have been shown to strongly inhibit their uptake.[12,18] Further, for quinolones, a correlation has been established between the affinity of these drugs to OmpF and their hydrophilicity, indicating that the enhanced antibacterial activity of the more hydrophilic newer generation is facilitated in presence of OmpF channels.[19]

The aim of this study was to elucidate the molecular details along the pathway of moxifloxacin and to understand the rate limiting contributions to moxifloxacin influx. As a measurement control, we selected a quinolone from a different class and generation, nalidixic acid, on the basis of its different hydrophobicity and expected pathway (for their respective structures, see panels C and D of Figure 1). Fluorescence anisotropy and quenching were used to determine the partition coefficients of the two investigated quinolones in liposomes.

Accelerated molecular dynamics (MD) simulations have revealed a putative translocation path for moxifloxacin through OmpF and the associated free energy surface (FES). Through the FES, binding sites and activation barriers can be identified, and these results can be successfully integrated and corroborated by the experimental data presented below.

High-resolution conductance measurements were performed on a single OmpF pore inserted in a planar lipid bilayer, and, as recently shown with the β-lactam antibiotic ampicillin,[20] the measurement of the drug residence time in the individual pores as blockages of the small ion current[21] made the determination of an association rate constant possible.[22] The results indicate that moxifloxacin interacts strongly with the interior of the OmpF channel, whereas nalidixic acid does not.

OmpF is a trimer and each monomer contains only two tryptophan residues (panels A and B of Figure 1), Trp214 at the lipid−protein interface and Trp61 at the trimer interface.[23] In contrast to β-lactams, quinolones can be used as resonance energy transfer (RET) acceptors for tryptophan fluorescence emission. Quenching studies and resonance energy transfer with tryptophanes as donors and quinolones as acceptors allowed the identification of their relative position. In addition, the MD provided a model for the strict interpretation of the fluorescence data at the atomic scale, discriminating the interactions between each of the two tryptophanes and moxifloxacin.

This work combined several complementary techniques to probe the microscopic details of the quinolone location and translocation through a lipid bilayer in presence of reconstituted OmpF. The results of the study clearly indicate that nalidixic acid, a more hydrophobic quinolone, is located on the lipid−protein interface and that moxifloxacin, a more hydrophilic quinolone, translocates through the OmpF lumen, with a pronounced binding site near Trp61. The collected reliable information at a microscopic level by these different complementary techniques can represent a valid tool of a bottom-up strategy to design new and more efficient antibiotics.

Results and Discussion

For the selection of moxifloxacin and nalidixic acid, we revisited the water/octanol partition coefficients[24,25] and measured their partition in a lipid environment. Fluorescence emission intensity and steady-state anisotropy revealed a partition coefficient[26] between the lipid and aqueous phases of $K_p = 232 \pm 63\ M^{-1}$ for moxifloxacin, whereas for nalidixic acid

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a higher partitioning in the lipid phase with $K_p = 1060 \pm 123$ M^{-1} was measured in agreement with previous water/octanol partitions. The coefficients suggest different possible translocation routes for the more hydrophilic versus more hydrophobic quinolone. Molecular dynamics (MD) simulations were performed to obtain a more detailed translocation process of moxifloxacin on the length- and time scale of the binding and translocation.

Molecular Modeling. A preliminary MD study was carried out to energetically characterize the cis and trans forms of moxifloxacin as a function of the dihedral angle separating the hydrophobic from the planar region. Quantum chemistry calculations of the optimized structures in the electronic ground state, performed with the package *Gaussian 03*,[27] showed a difference of ~7 kcal/mol with a preference for the trans form. The parametrization of the restrained electrostatic potential (RESP)[28] charges of the force-field was done using as a reference the electrostatic potential of the optimized trans conformer alone. As a further indication of the high stability of the trans conformer in the typical time considered here, metadynamics simulations[29] yielded a trans−cis barrier of the order of 17 kcal/mol (Supporting Information).

The moxifloxacin molecule is initially placed with its dipole matching the charged groups of the constriction region, with the $>NH_2+$ group close to Glu117 and the $-CO_2-$ interacting with Arg132, as for other zwitterionic antibiotics this was the preferred conformer.[30] After a standard MD relaxation at 300 K, moxifloxacin does not leave this initial location, confirming the favorable interaction between moxifloxacin and the charged amino acids of the constriction region. Starting from this structure, we performed metadynamics using as collective variables[29] the number of hydrogen bonds and the position of the center of mass of moxifloxacin with respect to the center of mass of the protein–detergent system projected on the Z axis of the porin, Z_{cm}. After a 13 ns simulation time, moxifloxacin translocated to the periplasmic space. The reconstructed free energy surface (FES) in the space of the two collective variables is reported in Figure 2, where each isoline corresponds to 1 kcal/mol. On the FES several minima are present. The minimum at $Z_{cm} \sim 7.5$ Å above the constriction zone and forming ~6 hydrogen bonds is the starting structure. During the translocation, moxifloxacin senses a second less-pronounced minimum at $Z_{cm} \sim 7.5$ Å with a loss of hydrogen bonds, before reaching a third one at $Z_{cm} \sim 3.5$ Å characterized by new pattern of hydrogen bonds. From this position, the molecule should overcome a barrier of ~ 9 kcal/mol to pass the constriction zone. Along this path, moxifloxacin maintains the trans geometry and turns the carboxylic acid group upward with respect to the Z axis of the porin, embedding its hydrophobic part in the hydrophobic pocket of the constriction zone, interacting with Glu117, Tyr40, Tyr310, and Val18. It is interesting to note that in this minimum ($Z_{cm} \sim 3.5$ Å), reached after 6 ns of metadynamics, the moxifloxacin dipole is still matching the electric field of the constriction region as in the starting conformer, with the amminic group near Glu117 and the carboxylic group near Arg132 (Figure 3). An additional standard

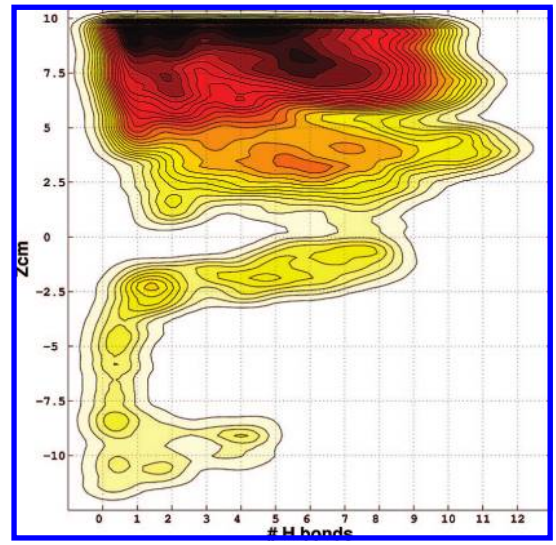

Figure 2. The free energy surface associated with the reaction pathway along the subspace of the two variables, displacement of the center of mass of moxifloxacin along the Z axis of the porin ($Z_{cm} = 0$ corresponds to the constriction zone), and number of hydrogen bonds formed. Each isoline corresponds to 1 kcal/mol.

MD simulation performed starting from this minimum (4.3 ns) showed a high stability of moxifloxacin, confirming strong interactions with the charged groups of the constriction region and the hydrophobic pocket.

The translocation path obtained from the metadynamics provides an atomistic perspective that can be useful to interpret the experimental results, whereas it can also be verified by experimental results, as discussed in the next sections.

High-Resolution Conductance Studies. To measure the interaction of the two antibiotics by a single-molecule method, a single OmpF trimer was reconstituted into a solvent-free planar lipid bilayer,[31] and its conductance of about 0.9 nS was measured in 150 mM KCl solution.

Addition of nalidixic acid to the bathing solution did not cause significant fluctuation in the ion current as measured in the applied voltage range (−100 mV to +100 mV) (Parts A and B of Figure 4). In contrast, on addition of moxifloxacin, conductance fluctuations were observed in the current output. Parts C and D of Figure 4 show that moxifloxacin produces time-resolved current interruptions characteristic of reversible blocking of one channel of the trimer, not present before the antibiotic addition. Control measurements to elucidate a possible channel asymmetry with respect to antibiotic translocation have been performed by single-sided addition − the difference in dwell times and number of events were within the overall measured error ranges.

Kinetic constants of moxifloxacin association to the OmpF channel may be obtained from the steady-state analysis of open channel probability and the measurement of the average residence time in the current−time recording.[32] The average residence time was determined to be 60 ± 30 μs at 5 mM moxifloxacin concentration by single-channel analysis of the current measurements in the time domain, fitting a single

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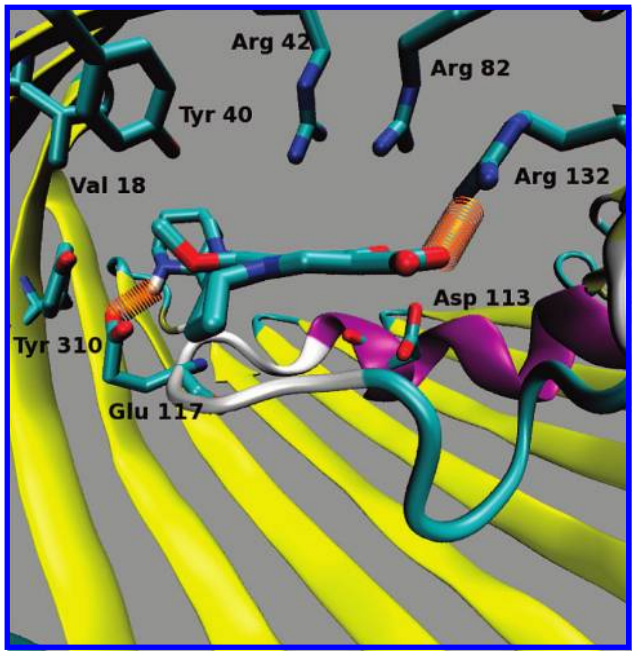

Figure 3. View from the extracellular side of moxifloxacin in the proposed binding site with the charged amino acids of the constriction region and the hydrophobic pocket (hydrogen bonds in orange).

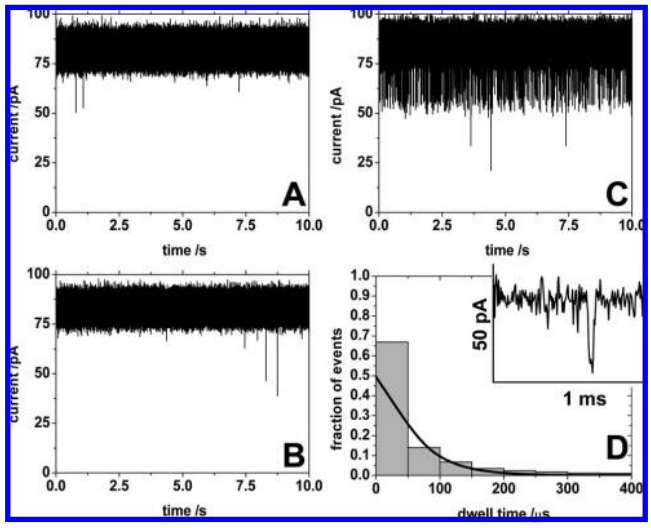

Figure 4. Current traces of a single OmpF trimer insertion into a solvent-free black lipid membrane, with a potential difference of +90mV applied, using a 30 kHz low-pass filter, in 150 mM KCl, 10 mM HEPES, pH 7.0: A) before antibiotic addition, B) 5 mM nalidixic acid added to the ground electrode (cis) side, C) 5 mM moxifloxacin added to the cis side. The dwell-time histogram corresponding to current blocking events in recording C) can be seen in D), with a single-monomer antibiotic blocking event inset (bars represent 1 ms and 50 pA).

exponential to a time histogram (part D of Figure 4), considering residual of fit and comparing 6 measurement runs, 10 000 events each.

Because of the low residence time of moxifloxacin, the current measurements needed to be undertaken at a high filter frequency, resulting in large apparent current noise and making an event count in the time domain difficult and with large uncertainty. Power-density analysis was applied[33] for a more precise

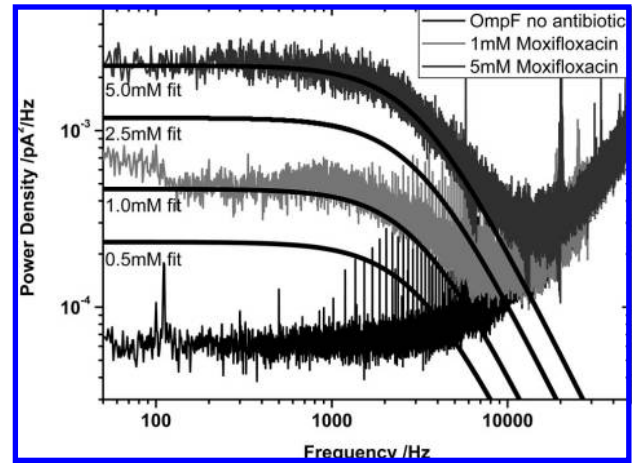

Figure 5. Example raw untreated power density spectra of current recordings of a single OmpF trimer insertion into a planar lipid membrane (−50mV applied voltage, in 150 mM KCl, 10 mM HEPES, pH 7.0), without antibiotic addition, and in the presence of 1 mM and 5 mM moxifloxacin in the cis chamber (ground electrode). Overlaid are Lorentzian distribution fits to the power spectra obtained at different moxifloxacin concentrations in the same measurement after subtraction of the background (OmpF without antibiotic).

visualization of the time correlation. Addition of a substrate binding to the channel in a double-markovian random process[34] generates an excess current noise whose power spectrum can be described by a single Lorentzian.[35] It has previously been shown that this noise can be used to obtain characteristic transport parameters and kinetic information.[36,37]

A spectrum can be fitted to a Lorentzian $S(f) = S(0)/(1 + (f/f_c)^2)$, where the corner frequency f_c gives the relaxation time constant and the kinetic constants of channel blocking in the case of symmetric substrate addition as:[33,35,38]

$$2\pi f_c = \frac{1}{\tau_{\text{relaxation}}} = k_{\text{on}}c + k_{\text{off}} \qquad (1)$$

where k_{off} is the cumulative off-rate toward both sides; k_{on} is the association rate, and c is the drug concentration. All of the data were considered after subtracting a background noise spectrum obtained from a conductance measurement after protein insertion with only bathing solution present, immediately before the addition of the antibiotics. Typical raw current noise spectra before background subtraction obtained by addition of different moxifloxacin concentrations on one side of the chamber are displayed in Figure 5, together with Lorentzian fits calculated after subtraction of background. The noise induced by moxifloxacin exceeded the background by up to 2 orders of magnitude at low frequencies at high moxifloxacin concentrations (above 5 mM). The addition of nalidixic acid did not result in a change in the spectrum in the entire frequency range measured (1 Hz to 100 kHz) at any of the applied voltages. It is possible to conclude that, even if nalidixic acid penetrates the OmpF pore, its residence time is below the microsecond time scale − details of a translocation process below this scale are currently not accessible with ionic current measurements

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but can be elucidated using spectroscopic techniques (below).

The chemical association of moxifloxacin with the channel can however be calculated to a high precision using the characteristic concentration dependent Lorentzian power-density distribution measured. In our case, because of the measured short residence time, the characteristic relaxation time is close to the average time of drug residence in the channel[20,21] $\tau_{residence} \approx \tau_{relaxation} \approx k_{off}^{-1}$. From these considerations, the fitted values for the residence time result in 52 ± 5 μs for 5 mM moxifloxacin, in agreement with the time-domain analysis. Fitting the dependence of the relaxation time on concentration a cumulative off-rate $k_{off} = 19 \pm 2$ ms^{-1} and cumulative on-rate $k_{on} = 320 \pm 30$ ms^{-1} M^{-1} could be calculated, resulting in a binding constant $K = k_{on}/k_{off} = 16.8 \pm 1.6$ M^{-1} of moxifloxacin to OmpF under symmetric antibiotic addition. The residence time of moxifloxacin is shorter than those obtained for well-binding β-lactam antibiotics but on the same time scale as for ampicillin (120 μs).[20]

The measured residence time is of the same order of magnitude as the time (low microseconds) needed to overcome the barrier of 9 kcal/mol, obtained from MD simulations, using transition-state theory.

Fluorescence Spectroscopy. To experimentally access more precise positional information of moxifloxacin and a possible association of nalidixic acid, we employed fluorescence spectroscopy. When antibiotic solution containing moxifloxacin or nalidixic acid is added to an OmpF containing proteoliposome or mixed-micelle suspension, a quenching of the tryptophan fluorescence is observed. The observed quenching can be due to a fluorescence resonance energy transfer (FRET) between the OmpF donor and the quinolone acceptor, as the emission spectra of OmpF and the absorption spectra of the quinolones overlap (Figure 6). The rate of energy transfer depends on the extent of spectral overlap, the quantum yield of the donor, the relative orientation of the donor and acceptor dipoles, and the distance between the donor and the acceptor.[39] Structural information from fluorescence energy transfer data can be obtained using Förster's theory in a 2D system.[40] The donor−acceptor distance at which energy transfer and spontaneous decay of the donor's excited-state are equally probable (the Förster radius) is given (in Å) by

$$R_0 = 9.78 \times 10^3 \left[k^2 \varphi_D n^{-4} \int_0^\infty I(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda\right]^{1/6} \qquad (2)$$

where k^2 is the orientation factor, ϕ_D is the donor quantum yield in the absence of the acceptor, n is the refractive index of the medium, $I(\lambda)$ is the normalized fluorescence spectrum, and $\varepsilon(\lambda)$ the molar absorption coefficient (in M^{-1} cm^{-1}).

The presence of two distinct tryptophanes per OmpF monomer does not allow the discrimination of the precise individual interactions. Instead, the putative translocation pathway indicated by the MD simulations may be used in conjunction with the experimentally obtained spectral overlap, to obtain the average energy transfer efficiency (E) between moxifloxacin and each of the two tryptophanes individually as

$$E = \frac{R_0^6}{R_0^6 + r^6} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \qquad (3)$$

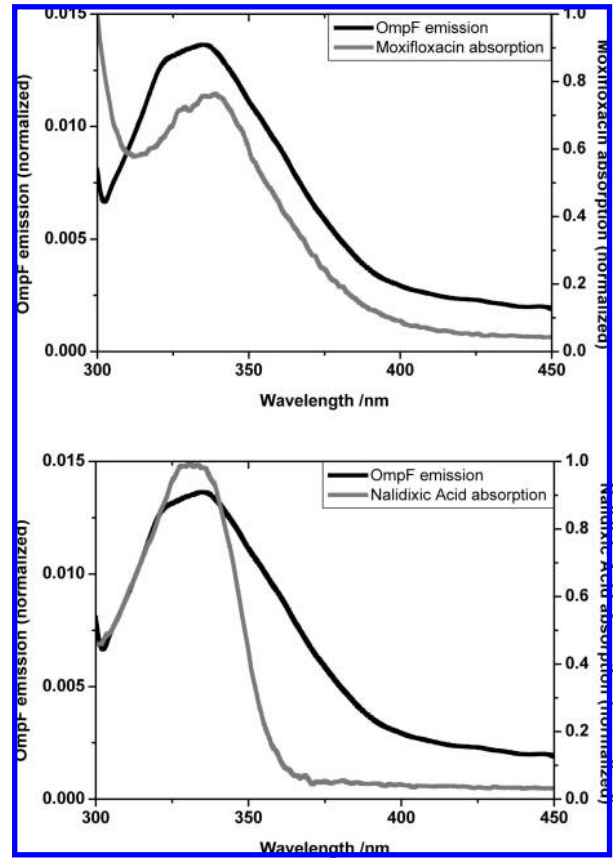

Figure 6. Overlap of OmpF emission and antibiotic absorption spectra. The OmpF emission has been normalized to an integral of unity, whereas the absorption curves have been normalized relative to their maximum value − this normalization convention straightforwardly results in the calculation of overlap the integral.

Table 1. Calculated Energy Transfer Efficiency between Moxifloxacin and the Tryptophanes from MD Simulations (eq 3)[a]

	Trp61	Trp214
E(ISO, $k^2 = {}^2/_3$)	0.94	0.70
E(MD-META, k^2 = calculated)	0.96	0.38
E(MD-BINDING, k^2 = calculated)	0.96	0.62

[a] Calculated using an isotropic orientation factor (first line), using orientation factors obtained from the MD simulation for the translocation trajectory (center), and using the simulation of moxifloxacin residing in the supposed binding site (Figure 3) (bottom).

where R_0 is the Förster radius from eq 2 (assuming n = 1.4),[41−43] and r is the donor−acceptor distance, extracted from MD simulation. The energy transfer efficiency increases with the donor−acceptor distance decreasing below the Förster radius. The values of E, calculated according to different protocols (below), were collected in Table 1. From the MD trajectory, the estimated Trp−moxifloxacin distances are 18 Å and 24 Å respectively for Trp61 and Trp214 (Figure 7), suggesting a higher interaction between moxifloxacin and Trp61. Using the standard[26] orientational assumption $k^2 = {}^2/_3$, which corresponds to a dynamically isotropic (or pseudoisotropic) regime of transfer, R_0 for a random array of donors and

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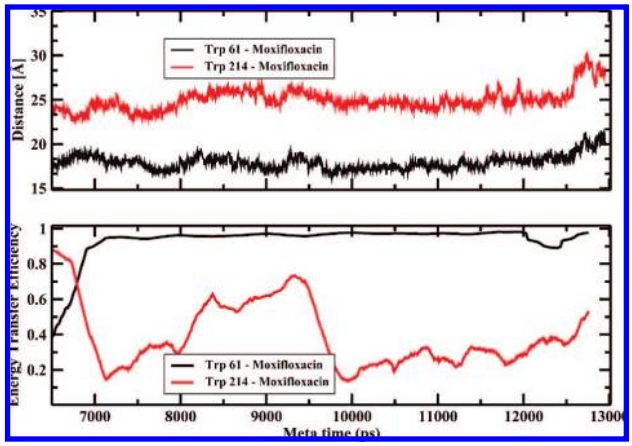

Figure 7. Relative distances of moxifloxacin from Trp61 and Trp214 as function of simulation time (top) and calculated energy transfer efficiency as by eq 3 (bottom).

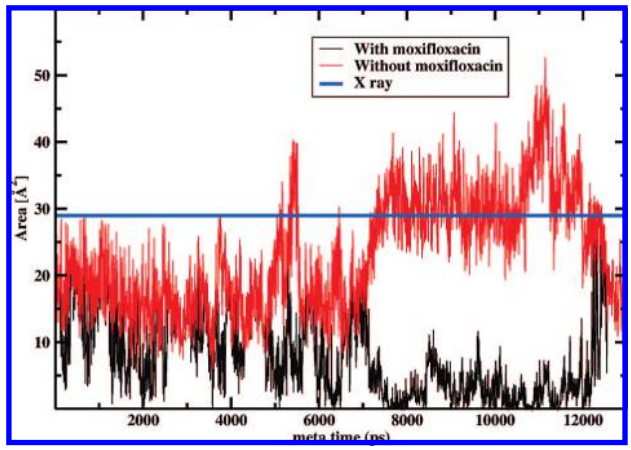

Figure 8. Calculated area available to a probe of radius 1.4 Å at the constriction region ($Z_{cm} = +1$ in Figure 2). Available area with moxifloxacin in the channel (black) and channel area not considering that part of it is obscured by moxifloxacin (red) along the metadynamics trajectory. The area available in the X-ray crystal structure is plotted for comparison in blue.

acceptors[44] is 28.8 Å for moxifloxacin and 25.4 Å for nalidixic acid. According to these results the observed quenching in the case of moxifloxacin could be a consequence of RET between either of the Trp donors and the acceptor. More specifically, the calculated E for this random orientation factor are 0.94 and 0.70, for Trp61 and Trp214, respectively, obtained by averaging over the metadynamics trajectory (from the binding site until the end) and denoting strong energy transfer for both tryptophanes (first line of Table 1).

The orientation factor is determined by the direction of the emission dipole of the donor (Trp) and the absorption dipole of the acceptor (moxifloxacin).[26] As moxifloxacin moves inside a confined geometry, the value of the orientation factor should not a priori coincide with the one obtained above for a random stereodistribution. From the trajectory predicted by MD, a more precise evaluation of the Förster radius is possible on the basis of an explicitly calculated orientation factor k^2. The average efficiencies of energy transfer obtained by an explicit calculation of the orientation factor are 0.96 for Trp61 and 0.38 for Trp214, indicating preferential energy transfer from Trp61 to moxifloxacin (central line of Table 1). The same preferred interaction with Trp61 is also evident from the calculated transfer efficiency of moxifloxacin when residing in the binding site (using an additional standard MD simulation of 4.3 ns), with $E = 0.96$ for Trp61 and $E = 0.62$ for Trp214 (bottom line in Table 1). In Figure 7, we report the distance of moxifloxacin to the tryptophanes together with the energy transfer efficiency over the metatrajectory from 6 to 13 ns, after moxifloxacin reached the minimum at Z $\sim$ 3.5 Å.

FRET measurements only indicate that both drugs interact with tryptophan residues. To attribute the energy transfer of each distinct tryptophan to the antibiotics, experimentally corroborating the MD results, the quenching of OmpF fluorescence by acrylamide and iodide was measured. In these quenching studies the Stern−Volmer plots display a downward curvature, showing a different accessibility of the two tryptophan residues to the quenchers. The quenching process is thus described by a modified Stern−Volmer equation:

$$F_0 - F = F_{0a} \cdot \left(\frac{K_a[Q]}{1 + K_a[Q]} \right) \tag{4}$$

where $F_0 - F$ refers to the change in fluorescence intensity on addition of the quencher, F_{0a} refers to the initial fluorescence in the absence of the quencher, and K_a is the Stern−Volmer constant of the accessible fraction of tryptophanes. The value of the fraction of the initial fluorescence that is accessible to the quencher (f_a) is easily obtained at saturation from eq 5:

$$f_a = \frac{F_{0a}}{F_0} \tag{5}$$

Considering all correction factors, the spectral changes observed after the addition of the quenchers to the samples containing OmpF (or OmpF and antibiotic) were treated by nonlinear graphical methods, using the wavelength of maximum fluorescence intensity.[19]

The quenching by acrylamide was unaffected by the lipid environment, whereas the quenching constant in presence of iodide ions (repelled by a negative charge) strongly decreased in the presence of negative 1,2-Dimyristoyl-sn-Glycero-3-Phospho-(1′-rac-Glycerol) (DMPG). The behavior suggests that the access of acrylamide to Trp61 in the core of the trimer interface is unaffected by the surrounding lipid, and the preferential access of iodide is to Trp214 near the protein−lipid interface. This is further confirmed by the higher f_a value in presence of acrylamide (Table 2), as Trp61 has a higher fluorescence intensity, because it is located in a more hydrophobic environment.

As shown in Table 2, in the presence of nalidixic acid, the Stern−Volmer constant for iodide quenching is strongly reduced, whereas that for acrylamide remains unaffected, suggesting an interaction of nalidixic acid with the Trp214, on the exterior of the porin. This putative pathway through the lipid membrane correlates with no interaction in the channel interior observed by conductance measurements. In contrast, the acrylamide quenching is strongly modified by moxifloxacin suggesting an interaction of the antibiotic with Trp61 in the interior of the channel, modifying the environment of the channel lumen. In the presence of moxifloxacin, the Stern−Volmer constant for iodide is also slightly increased, which can be explained either by a possible slight destabilization of the lipid packing

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Table 2. Stern−Volmer Constants and Accessible Fraction of the Fluorescence Quenching of the OmpF Tryptophanes (inserted in DMPC) by Acrylamide and Iodide, with and without the Addition of 27.9 μM of Each of the Two Antibiotics

	no drug		nalidixic acid		moxifloxacin	
	K_a (M^{-1})	f_a	K_a (M^{-1})	f_B	K_B (M^{-1})	f_a
acrylamide/DMPC	3.8 (±0.1)	0.6 (±0.1)	3.5 (±0.2)	0.6 (±0.1)	5.1 (±0.4)	0.6 (±0.2)
I^-/DMPC	2.8 (±0.2)	0.4 (±0.1)	1.5 (±0.3)	0.5 (±0.1)	3.1 (±0.2)	0.4 (±0.1)

with moxifloxacin perturbing one monolayer leaflet[45] facilitating iodide access to Trp214, or by a conformational change of OmpF accommodating the translocating antibiotic, exposing the Trp214 closer to the bilayer surface.

A conformational change is verified from the MD results. To locate the state corresponding to the closure of the pore to ionic conductance measured in the lipid bilayer experiments, the accessible area to a probe of radius 1.4 Å along the *Z* axis was calculated. Figure 8 shows the calculated accessible area at the constriction region ($Z = +1$ Å with respect to the OmpF center of mass, Figure 2) for the whole metadynamics trajectory. After 6 ns, when moxifloxacin reaches the supposed binding site, the area available is almost zero, explaining ionic current blockages. The actual cross-sectional area of the pore is distended exactly when moxifloxacin reaches the binding site (Figure 8). This pore enlargement indicates a rearrangement at the constriction region to accommodate moxifloxacin, resulting in a conformational change, and possibly explains the slight increase of the Stern−Volmer constant for iodide.

Conclusion

Molecular modeling of moxifloxacin translocation through the OmpF channel determined a strong affinity site of the antibiotic in the channel lumen with its amminic group near Glu117 and its carboxylic group near Arg132. Artificial lipid bilayer measurements confirmed a binding of the moxifloxacin molecule inside the OmpF with a residence time of 52 ± 5 μs, on the same order of magnitude as the time necessary to overcome the simulated energy barrier to the constriction zone of 9 kcal/mol.

Judging from the translocation trajectories extracted from the atomistic MD simulation, resonance energy transfer should occur between the Trp residues in OmpF and the moxifloxacin − this was verified by FRET measurements. Exact intermolecular distances and explicit orientation factors obtained from MD simulation were used to distinguish the energy transfer efficiency of the two distinct tryptophanes, Trp 61 and Trp214 − this allowed us to further confirm our simulation by the measurement of modifications in Stern−Volmer constants of two quenchers (iodide and acrylamide) specific to the two Trp residues.

Analytical considerations of the translocation probability of particles through a channel show that the molecule-channel interactions are important in determining the flux.[46−48] The measured kinetic rates can be related to the flux.[22] For a symmetric on-rate, as has been measured for moxifloxacin, the flux can be estimated with the antibiotic present only on the extracellular side[22] as

$$J = \frac{Kk_{\text{off}}}{4 + 2K\Delta c}\Delta c \qquad (6)$$

The net flux J (in molecules/s) is at a low concentration (nonsaturated condition) proportional to the concentration gradient Δc. It should be noted that for a strong binding the permeation is limited by the off-rate and that for a large gradient Δc the flux is independent of the concentration gradient. More sophisticated models have been suggested that include detailed binding parameters. However, the above simplified model gives a reasonable estimate for the molecular flux. Inserting experimental values for $k_{\text{off}} = 19 \cdot 10^3$ s^{-1} and $K = 16.8$ M^{-1} gives about 0.1 molecules/s per monomer at a 1 μM (clinically relevant[49]) concentration gradient. This value is on the order of magnitude required to cause bacterial death[18] given the approximately 10^5 channels in the bacterium.[50]

On the basis of the molecular dynamics, it should be possible to tune the flux of a molecule by changing its intermolecular interactions if the binding sites and passage routes are known. The multidisciplinary approach presented here combining experimental techniques with MD simulations to create a more accurate picture is extremely attractive for explaining the molecular process of antimicrobial translocation. This may be the basis for the design of antibiotics by utilizing ad hoc molecular transport properties that enhance the antibiotic flux through porins.

Penetration rate into the periplasmic space is just one parameter modulating antibacterial accumulation,[51] and the prevention of accumulation is just one of the ways of bacterial resistance to antimicrobials.[52] However, less specific resistance mechanisms such as permeability barriers may become increasingly significant in the development of new more efficient wide-spectrum antibiotics and assessing multicomponent antimicrobial resistance.

Experimental Section

Moxifloxacin was received as a gift from Bayer (Leverkusen, Germany) and also obtained from Sequoia Research Products (Pangbourne, UK). Nalidixic acid was obtained from Sigma Aldrich (Buchs, Switzerland). All chemicals used were from Merck (Darmstadt, Germany) (pro analysi), except KCl, KOH, HCl and *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) (Applichem, Darmstadt, Germany), *n*-octylpolyoxyethylene (octyl-POE) (Bachem, Bubendorf, Switzerland), chloroform, hexadecane, and hexane (Fluka, Buchs, Switzerland) and all lipids from Avanti Polar Lipids (Alabaster, AL). Doubly distilled and deionized water was used to prepare all solutions.

OmpF was purified from *E. coli*, strain BL21 (DE3) Omp8, following published procedures.[53,54] OmpF concentration was

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estimated using the bicinchoninic acid protein assay against bovine serum albumin as standard.[55,56]

Conductance Measurements. The antibiotic stock solutions for conductance measurements were made at the final concentration of 10.0 mM in 150 mM KCl, 10 mM HEPES (pH 7.0).

The dissolution of the antibiotics in 150 mM KCl was facilitated by KOH addition and subsequent pH adjustment.[57−59] The pH of the solution was adjusted after the preparation of the stock solution and verified from samples taken at every stage of the experiment.

For the nalidixic acid, sodium-salt control experiments were performed to ascertain that the small percentage of sodium ion contamination does not change the system or protein properties − no such effect was found. Planar lipid bilayers were formed with the monolayer opposition technique,[31] across an 80 μm diameter circular aperture in a 25 μm thick Polytetrafluoroethylene film (Goodfellow, Cambridge, UK) being tightly glued between two Delrin chambers manufactured in-house, each containing 250 μL of an aqueous bathing solution, through the layering of 5 μL of a 2.5 mg/ml solution of 1,2-Diphytanoyl-sn-Glycero-3-Phosphatidylcholine (DPhPC) in a 1:9 mixture of chloroform to hexane on the buffer surface on each side. The apparatus was shielded from external electromagnetic fields by a doubly isolated Faraday cage and from acoustic interference by an isolating closet on a vibration-resistant table made in house. Control measurements were performed with *E. coli* polar lipid extract to ascertain the biological relevance of the results with respect to the lipid used − no significant differences were observed. Two microliters of wild-type OmpF porin from a 1.5 ng/ml solution in 150 mM KCl with 1% octyl-POE was added to the cis side compartment (contacted by the ground electrode). Incorporation was achieved by stirring after addition and applying a 150−250 mV transmembrane voltage.

OmpF inserts into artificial bilayers in a strictly ordered manner − there is a strong indication that the porin inserts with periplasmic loop first, that is, if the protein is added to the cis side, the normally periplasmic side is on the trans side, the extracellular side is on the cis side.[60]

Electrical recordings were made through a pair of Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL), attached to an Axon Instruments 200B amplifier with a capacitive headstage, digitized by an Axon Digidata 1440A digitizer, computer controlled by Clampex 10.0 software (all by Axon Instruments, Foster City, CA).

The data was filtered by an analogue low-pass 4-pole Bessel filter at 100 kHz, and digitally sampled at 500 kHz. For spectral analysis the data was used raw, for event detection subsequently down-filtered to 30 kHz by a digital low-pass 8-pole Bessel-filter, provided in the *Clampfit 10.0* software (Axon Instruments, Foster City, CA).

Data analysis and calculation of spectral density was carried out on the *Clampfit 10.0* software and on an in-house program written using the *Matlab* package (Mathworks, Natick, MA).

The concentration of antibiotic was adjusted by solution exchange in the appropriate compartments, not changing the total aqueous volume. At every addition stage the concentration was corroborated by subsequent UV−vis adsorption from liquid samples withdrawn after measurement. After the antibiotic addition, the solution was homogenized by a short initial stirring and a consecutive 15 min incubation leaving time for spontaneous diffusion.

Molecular Dynamics. In the molecular dynamics simulations, the system is modeled by a single monomer of OmpF (pdb-id: 2OMF, resolution 2.4 Å)[61] embedded in a hydrophobic environment of detergent molecules (lauryl dimethyl amine oxide, LDAO) and solvated with 7600 water molecules. Both experimental and theoretical investigations on OmpF have pointed out the mutual independence of the three subunits (no cooperativity between the three monomers) for ion[62] and small molecule transport.[63,64] The absence of cooperativity and the rigidity of the structure reduce the importance of low-frequency breathing modes in determining the channel dynamics and make MD simulations a feasible and reliable tool to investigate antibiotic translocation at the microscopic level. The detergent, generally used to crystallize membrane proteins, provides a natural hydrophobic environment that prevents direct interactions between the external protein surface and water. The preparation of the system followed the protocol described elsewhere,[65] all residues being in their standard ionization state corresponding to pH 7 except for Glu-296 and Asp-312, which were protonated; counter-ions were introduced to neutralize protein charges. We used the AMBER force field for OmpF, the detergent,[66] and the antibiotics, TIP3P for water. All simulations were performed, after relaxation, at constant volume and temperature using state-of-the-art software.[67]

Details of the metadynamics algorithm to investigate rare events have been described previously.[29] Briefly, to overcome the time scale problem we added artificially a time-dependent Gaussian term to modify the underlying free energy during the simulation. This creates a component to the forces that prevents the molecule from revisiting the same place and drives the molecule through the channel. This additional term acts only on a few slowly changing variables that describe the translocation process, making them faster on the MD simulation time scale. For the present study, two slow variables were selected − the position of the drug center of mass (c.o.m.) along the z axis, that is, the pore axis, and the number of hydrogen bonds between the drug and the channel. More specifically, the following setup of the metadynamics simulations was adopted for the system: i) a wall was placed at 9.5 Å above the constriction zone to avoid the exit of moxifloxacin from the porin; ii) the Gaussians were added every 4 ps; iii) the height of every Gaussian is 2 kJ/mol; iv) the spreads of the Gaussians in the spaces of the hydrogen bond number and of the com z-position were 0.5 and 0.25 Å, respectively.

Fluorescence Spectroscopy. All solutions for fluorescence measurements including antibiotics solutions and proteoliposomes suspensions were prepared with 10 mM HEPES buffer (0.1 M NaCl; pH 7.4).

Spectrophotometric and fluorescence measurements were performed in a Varian spectrofluorometer, model Cary Eclipse, equipped with a constant-temperature cell holder (Peltier single cell holder). All of the spectra were recorded at 37 °C, under constant stirring, with a slit width of excitation and emission of 10 nm, from 305 to 400 nm for emission and 290 nm for excitation. Inner filter effects and dilution of the solution were accounted for.

OmpF proteoliposomes were prepared by direct incorporation into preformed liposomes of 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DPMC) or *E. coli* polar lipid extract, by well

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established methodology,[68−70] as follows. To an adequate volume (∼2.6 mL) of liposome suspension (∼2.0 mM) in HEPES buffer, prepared according usual procedures,[71,72] a determined quantity of OmpF (0.25 mg) was added in a HEPES buffer solution with 0.4% of octyl-POE. The molar ratio between lipid/protein was always near 1000, and the total volume of the mixture assured a final concentration of octyl-POE lower than value of its CMC (0.23%). After thorough homogenization of all constituents by gentle stirring, the mixture was incubated 15 min at room temperature followed by 1 h on ice. The detergent was then adsorbed onto SM2 Bio-Beads (Bio-Rad Laboratories, Hercules, CA) at a concentration of 0.2 g of Bio-Beads/ml, by gently shaking the suspension for a period of 3 h. After this time, a second portion of the same amount of Bio-Beads was added, and the suspension was again shaken for another 3 h, followed by a gentle removal of the proteoliposomes via decanting the Bio-Beads. The suspension of proteoliposomes was submitted to ultracentrifugation (80,000*g*, 4 °C, 2 h) to remove any traces of protein not inserted. After this procedure, the supernatant was rejected and the pellet suspended in HEPES buffer. Finally, the proteoliposomes were sequentially extruded through 200 and 100 nm polycarbonate membranes (Nucleopore), to guarantee the homogeneity of the size distribution in the suspension.

Quinolone fluorescence quenching studies were achieved by successive additions of a constant volume (10 μL) of quinolones solution to the cuvette (final concentration range: 0−38 μM) containing a constant amount of OmpF (∼0.45 μM) incorporated in the liposomes. The acrylamide and iodide fluorescence quenching studies were achieved by successive additions of a constant volume (10 μL) of acrylamide or KI solutions (5.0 M) to the cuvette (final concentration range: 0.0−0.5 M) containing a constant amount of OmpF (∼0.45 μM), in mixed micelles or inserted in liposomes, in the absence or in the presence of a certain constant quantity (final concentration between 26−30 μM) of quinolone. The first UV−vis and fluorescence spectrum is taken before the additions (with only protein suspension or protein suspension plus constant amount of quinolone), and then after each addition fluorescence spectrum and absorption at the excitation wavelength (290 nm) are obtained. Equation fitting was performed using the *Origin 6.1* software (OriginLab, Northampton, MA).

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Supporting Information Available: Free-energy profile of the barrier separating the cis and trans conformer form of moxifloxacin in gas phase and water obtained from metadynamics calculations. Calculated area of the porin available to a probe radius of 1.4 Å along the entire porin *Z* axis with and without moxifloxacin. This material is available free of charge via the Internet at http://pubs.acs.org.

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