Lack of Interaction of Fluoroquinolones with Lipopolysaccharides

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Fluoroquinolones are known to chelate with di- and trivalent cations, and it has accordingly been claimed that they perturb the integrity of the outer membrane (OM) of gram-negative bacteria. So far, chelation has not been assessed in biologically relevant test systems. Therefore, we investigated the interaction of ciprofloxacin and moxifloxacin in the absence and presence of Mg^{2+} with whole bacteria and isolated lipopolysaccharide (LPS) from various rough mutant strains of *Salmonella enterica* chemotypes by applying different biophysical techniques. We found that the fluoroquinolones did not disturb the integrity of the OM and neither were incorporated into LPS monolayers nor displaced Ca^{2+} from LPS monolayers, suggesting that chelation of fluoroquinolones with divalent cations does not contribute to the antibacterial effect of fluoroquinolones.

Fluoroquinolones have a tendency to chelate di- and trivalent cations (12, 13, 16, 18, 19, 26, 30, 31, 36). Therefore, it is tempting to speculate that fluoroquinolones chelate the magnesium that is associated with lipopolysaccharide (LPS) and maintains the integrity of the outer membrane (OM) (16). Chelation of magnesium would then create hydrophobic patches in the OM of gram-negative bacteria through which quinolones diffuse (4). This hypothesis of self-promoted uptake of quinolones has been disputed (9, 22), and it was demonstrated that fluoroquinolones may also be translocated via the F porin channel (5) or may diffuse through bilayers (11, 25).

So far, chelation was demonstrated only in physicochemical assays, with only the fluoroquinolone and di- or trivalent cations present in the test system (14, 22; S. Lecomte, C. Coupry, and M. T. Chenon, 32nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 785, 1992), but has not been assessed in biologically relevant test systems. Therefore, we investigated the interaction of ciprofloxacin, moxifloxacin, and trovafloxacin in the absence and presence of Mg^{2+} with whole bacteria from various rough (Re) mutant strains of *Salmonella enterica* and with the respective isolated LPS chemotypes by applying different biophysical test systems (reconstituted OM of gram-negative bacteria as planar asymmetric bilayers, monolayers, and liposomes).

The investigations of this study revealed that the fluoroquinolones did not influence the integrity of the OM of gram-negative bacteria. Thus, neither a direct influence on the permeability and fluidity of the lipid bilayer nor interaction with the divalent cations stabilizing the OM was observed.

A Langmuir film balance equipped with a Wilhelmy system (Munitech, Munich, Germany) was used to test whether fluoroquinolones present in an aqueous subphase are incorporated into Re LPS monolayers at a water-air interface. The monolayers were spread at 20°C from 1 mM Re LPS chloroform-methanol (9:1, vol/vol) solutions on a subphase (1, 8, 15, 21). The respective quinolones were added to the subphase to pro-

duce different concentrations (0 to 0.4 mM) after the monolayer had been equilibrated at the biologically relevant lateral pressure of 25 mN m⁻¹. The relative change in the monolayer area at constant pressure with time after the addition of the drug to the subphase and in the absence and presence of Mg^{2+} is given in Fig. 1. For moxifloxacin, a slight increase in the monolayer area up to an equilibrium value of 110% with respect to the control was reached about 30 min after addition of the drug. The presence of 5 mM $MgCl_2$ in the subphase at the same time inhibited this incorporation almost completely. Gentamicin at the same concentration did not lead to an increase in monolayer area.

The ability of the quinolones to displace divalent Ca^{2+} ions from LPS monolayers was investigated by utilizing the characteristics of the low-energy β^- radiation of $^{45}Ca^{2+}$. A beta counter (gas ionization detector LB124; Berthold, Wildbad, Germany) placed immediately above the LPS monolayer is able to detect only beta radiation from $^{45}Ca^{2+}$ ions within the LPS monolayer at the water-air interface (beta particles emitted by $^{45}Ca^{2+}$ ions present in the subphase are absorbed by water), allowing determination of the Ca^{2+} concentration in the LPS monolayer (17). Up to a concentration of 2×10^{-4} M in the subphase, none of the fluoroquinolones tested was able to displace Ca^{2+} from the Re LPS monolayer (data not shown).

By adsorption to or insertion into the membrane, membrane-active substances may modify the surface charge density and, with that, the surface potential of LPS aggregates or intact bacteria. This can be monitored by measuring the ζ potential (3) of the aggregates and bacteria in the absence and presence of drugs. ζ potential determination was performed with a 90 eta-Sizer 4 (Malvern Instruments, Herrsching, Germany). Up to a fluoroquinolone-LPS molar ratio of 1:1, ciprofloxacin, moxifloxacin, and trovafloxacin induced no significant change in the ζ potential. Experiments with intact bacteria of the respective Re mutant strains confirmed the results (data not shown). (Gentamicin caused a steep concentration-dependent increase in ζ potential from approximately -55 to 0 mV beginning at a concentration of 100 μ M and reaching a plateau at concentrations of greater than 250 μ M.)

A possible influence of the fluoroquinolones on the state of order of the acyl chains within the hydrophobic region of LPS

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FIG. 1. Relative (Rel.) changes in the area of Re LPS monolayers (at 25 mN m^{-1}) with time after the addition of moxifloxacin and gentamicin, respectively, to the subphase (100 mM KCl, 5 mM HEPES, pH 7, 20°C). The final drug concentration in the subphase was 0.1 mM.

aggregates was investigated by Fourier transform infrared spectroscopy (Nicolet 5-DX; Nicolet Instruments, Offenbach, Germany). The peak positions of the symmetric stretching vibration of the methylene groups, $\nu_s(\text{CH}_2)$, of the different LPS suspensions (10^{-2} M), which is a measure of acyl chain order (2, 20), were monitored every 2°C from 15 to 60°C at two different concentrations of each quinolone and the respective controls. Furthermore, the influence of the drugs on the antisymmetric stretching vibration of the phosphate groups in the hydrophilic head group was measured. In contrast to gentamicin, the quinolones did not change the phase transition temperature or the state of order of any of the LPS chemotypes, even up to molar quinolone-LPS ratios of 3:1 (data not shown). Furthermore, the Fourier transform infrared spectroscopic measurements of the antisymmetric stretching vibration of the phosphate groups (between $1,300$ and $1,200 \text{ cm}^{-1}$) in the hydrophilic head group of LPS revealed no significant changes when fluoroquinolones were added to the LPS aggregates (data not shown), indicating that interaction with the phosphate groups is also negligible.

Asymmetric planar bilayer reconstitution of the OM of gram-negative bacteria and electrical measurements were performed as described earlier (23, 28). Intact lipid bilayers are characterized by very low electrical conductance ($<10^{-11} \Omega$). Interactions with the membrane, resulting in transient or permanent disturbances, will lead to measurable increases in the transmembrane current (35). The fluoroquinolones, added to the subphase of the endotoxin side, did not alter the electrical conductance of the lipid bilayer, even at concentrations of up to $5 \times 10^{-4} \text{ M}$ (data not shown). For comparison, polymyxin B, which is known to form micellar structures within lipid bilayers, causes transient fluctuations in electrical conductivity at concentrations as low as $2.7 \times 10^{-7} \text{ M}$ (10, 27, 34).

From these results, it can be concluded first that the fluoroquinolones cause neither permanent nor transient lesions in the lipid bilayer representing the lipid matrix of the OM, even at concentrations of roughly 50 times the maximal level in serum during therapy (29). Second, fluoroquinolones interact only weakly with LPS molecules. Similarly, the experiments with the Langmuir film balance demonstrate that moxifloxacin is incorporated into the LPS monolayer at the air-water interface from the aqueous phase at a physiologically relevant pressure to only a small extent and this incorporation is even inhibited by the presence of 5 mM MgCl_2 . Also, no displacement of Ca^{2+} from the LPS monolayer by the fluoroquinolones could be observed. In all of the experiments in which different LPS chemotypes were utilized, no significant differences in the results were observed.

These data are in agreement with flow cytometric monitoring of ciprofloxacin-induced loss of viability with fluorescent probes. Although viable counts of ciprofloxacin-exposed *Escherichia coli* bacteria decreased continuously over time, there was little change in cell-associated fluorescence; the low fluorescence likely resulted from a reduction in the number of binding sites or from the lack of an effect on membrane integrity (24, 33). However, changes in cell-associated fluorescence were observed upon exposure to gentamicin, although the rapid loss of viability of *E. coli* was not paralleled by an equally rapid increase in fluorescence intensity (24).

Similarly, the data of this study indicate that gentamicin affected the ζ potential of both LPS aggregates and intact bacteria, influenced the phase behavior of LPS, and increased the transmembrane current. However, gentamicin was not incorporated into the LPS monolayer. These results indicate, in agreement with previous findings (6, 7, 32), that gentamicin

causes lesions in the lipid bilayer probably by displacing Ca^{2+} from it.

Thus, chelation of fluoroquinolones with divalent cations seems not to contribute to the antibacterial effect of fluoroquinolones by interfering with the integrity of the OM. Vice versa, divalent cations of the OM do not interact with the fluoroquinolones and, thus, do not decrease their antibacterial activity.

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REFERENCES

- Blume, A. 1979. A comparative study of the phase transition of phospholipid bilayers and monolayers. *Biochim. Biophys. Acta* **557**:32–44.
- Brandenburg, K., S. Kusomoto, and U. Seydel. 1997. Infrared spectroscopic conformational study of synthetic lipid A part structures, p. 329–330. In P. Carmona, R. Navarro, and A. Hernanz (ed.), *Spectroscopy of biological molecules: modern trends*. Kluwer Academic Press, Dordrecht, The Netherlands.
- Cafiso, D., A. McLaughlin, S. McLaughlin, and A. Winiski. 1989. Measuring electrostatic potentials adjacent to membranes. *Methods Enzymol.* **171**:342–364.
- Chapman, J. S., and N. H. Georgopapadakou. 1988. Routes of quinolone permeation in *Escherichia coli*. *Antimicrob. Agents Chemother.* **32**:438–442.
- Chevalier, J., M. Mallea, and J. M. Pages. 2000. Comparative aspects of the diffusion of norfloxacin, cefepime and spermine through the F porin channel of *Enterobacter cloacae*. *Biochem. J.* **348**:223–227.
- Dalhoff, A. 1983. Studies on the action of aminoglycosides on bacterial membranes. *Zentbl. Bakteriol. Mikrobiol. Hyg. A* **253**:427–431.
- Dalhoff, A. 1987. Pleiotropic actions of aminoglycosides. *Antibiot. Chemother.* **39**:182–204.
- Galanos, C., O. Lüderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9**:245–249.
- Hancock, R. E. W. 1997. Peptide antibiotics. *Lancet* **349**:418–422.
- Hancock, R. E. W., S. W. Farmer, Z. Li, and K. Poole. 1991. Interaction of aminoglycosides with the outer membranes and purified lipopolysaccharide and OmpF porin of *Escherichia coli*. *Antimicrob. Agents Chemother.* **35**:1309–1314.
- Hirai, K., H. Aoyama, T. Irikura, S. Iyobe, and S. Mitsuhashi. 1986. Difference in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. *Antimicrob. Agents Chemother.* **29**:535–538.
- Khan, A. A., T. R. Slifer, F. G. Araujo, Y. Suzuki, and J. S. Remington. 2000. Protection against lipopolysaccharide-induced death by fluoroquinolones. *Antimicrob. Agents Chemother.* **44**:3169–3173.
- Khan, A. A., T. R. Slifer, and J. S. Remington. 1998. Effect of trovafloxacin on production of cytokines by human monocytes. *Antimicrob. Agents Chemother.* **42**:1713–1717.
- Lecomte, S., M. H. Baron, M. T. Chenon, C. Coupry, and N. J. Moreau. 1994. Effect of magnesium complexation by fluoroquinolones on their antibacterial properties. *Antimicrob. Agents Chemother.* **38**:2810–2816.
- Lindner, B. 2000. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of lipopolysaccharides. *Methods Mol. Biol.* **145**:311–325.
- Loubeyere, C., J. Desnottes, and N. Moreau. 1993. Influence of sub-inhibitory concentrations of antibacterials on the surface properties and adhesion of *Escherichia coli*. *J. Antimicrob. Chemother.* **31**:37–45.
- Lüllmann, H., H. Plösch, and A. Ziegler. 1980. Ca replacement by cationic amphiphilic drugs from lipid monolayers. *Biochem. Pharmacol.* **29**:2969–2974.
- Ma, H. H. M., F. C. K. Chiu, and R. C. Li. 1997. Mechanistic investigation of the reduction in antimicrobial activity of ciprofloxacin by metal cations. *Pharm. Res.* **14**:366–370.
- Macias Sanchez, B., M. Martinez Cabarga, A. Sanchez Navarro, and A. Dominguez-Gil Hurlé. 1994. A physico-chemical study of interaction of ciprofloxacin and ofloxacin with polyvalent cations. *Int. J. Pharm.* **106**:229–235.
- Mantsch, H. H., and R. N. McElhaney. 1991. Phospholipid phase transitions in model and biological membranes as studied by infrared spectroscopy. *Chem. Phys. Lipids* **57**:213–226.
- Marcelja, S. 1974. Chain ordering in liquid crystals. II. Structure of bilayer membranes. *Biochim. Biophys. Acta* **367**:165–176.
- Marshall, A. J. H., and L. J. V. Piddock. 1994. Interaction of divalent cations, quinolones and bacteria. *J. Antimicrob. Chemother.* **34**:465–483.
- Montal, M., and P. Mueller. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA* **69**:3561–3566.
- Mortimer, F. C., D. J. Mason, and V. A. Gant. 2000. Flow cytometric monitoring of antibiotic-induced injury in *Escherichia coli* using cell-impermeant fluorescent probes. *Antimicrob. Agents Chemother.* **44**:676–681.
- Nikaido, H., and D. G. Thanassi. 1993. Penetration of lipophilic agents with multiple protonation sites into bacterial cells: tetracyclines and fluoroquinolones as examples. *Antimicrob. Agents Chemother.* **37**:1393–1399.
- Okazaki, O., T. Kurata, and T. H. Tachizawa. 1988. Studies on the mechanism of PK interaction of aluminium hydroxide, an antacid, with new quinolones in rats. *Xenobiot. Metab. Dispos.* **3**:387–394.
- Schröder, G., K. Brandenburg, and U. Seydel. 1992. Polymyxin B induces transient permeability fluctuations in asymmetric planar lipopolysaccharide/phospholipid bilayers. *Biochemistry* **31**:631–638.
- Seydel, U., G. Schröder, and K. Brandenburg. 1989. Reconstitution of the lipid matrix of the outer membrane of Gram-negative bacteria as asymmetric planar bilayer. *J. Membr. Biol.* **109**:95–103.
- Stass, H., and D. Kubitz. 1999. Pharmacokinetics and elimination of moxifloxacin after oral and intravenous administration in man. *J. Antimicrob. Chemother.* **43**(Suppl. B):83–90.
- Teixeira, M. H., L. F. Vilas-Boas, V. M. S. Gil, and F. Teixeira. 1995. Complexes of ciprofloxacin with metal ions contained in antacid drugs. *J. Chemother.* **7**:126–132.
- Tuncel, T., and N. Bergisadi. 1992. *In vitro* adsorption of ciprofloxacin hydrochloride on various antacids. *Pharmazie* **47**:304–305.
- Van Bambeke, F., M. P. Mingeot-Leclercq, R. Brasseur, P. M. Tulkens, and A. Schanck. 1996. Aminoglycoside antibiotics prevent the formation of non-bilayer structures in negatively-charged membranes. Comparative studies using fusogenic (bis(beta-diethylaminoethylether)hexestrol) and aggregating (spermine) agents. *Chem. Phys. Lipids* **79**:123–135.
- Wickens, H. J., R. J. Pinney, D. J. Mason, and V. A. Gant. 2000. Flow cytometric investigation of filamentation, membrane patency, and membrane potential in *Escherichia coli* following ciprofloxacin exposure. *Antimicrob. Agents Chemother.* **44**:682–687.
- Wiese, A., M. Munstermann, T. Gutsmann, B. Lindner, K. Kawahara, U. Zahring, and U. Seydel. 1998. Molecular mechanisms of polymyxin B-membrane interactions: direct correlation between surface charge density and self-promoted transport. *J. Membr. Biol.* **162**:127–138.
- Wiese, A., and U. Seydel. 2000. Electrophysiological measurements on reconstituted outer membranes. *Methods Mol. Biol.* **145**:355–370.
- Zupancic, M., and P. Bukovec. 1996. Complexation of magnesium (II) with ciprofloxacin and norfloxacin. *Acta Pharm.* **46**:221–228.