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Brief Article

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Probing the Interactions of Macrolide Antibiotics with Membrane-Mimetics by NMR Spectroscopy

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Supporting Information

ABSTRACT: Interactions of macrolide antibiotics with biological membranes contribute to their bioavailability but are also involved in the formation of phospholipidosis, which is caused by the inhibition of phospholipase A_1 activity. We determined the interaction strength and localization of macrolide antibiotics with membrane-mimetics. Macrolides bind to membrane-mimetics with the positively charged amino groups being close to the micelle surface and thereby protect the lipids from being degraded by phospholipase A_1 rather than inhibiting the enzyme.

INTRODUCTION

Macrolide antibiotics, such as erythromycin and azithromycin, have been in widespread clinical use for over 50 years and are effective against Gram-positive and certain Gram-negative microorganisms.[1] They consist of a 14- or 15-membered polyketide ring and two attached monosaccharide moieties. The first characterized macrolide antibiotic, erythromycin, was discovered in 1952 in *Saccharopolyspora erythrea* and several semisynthetic analogues followed. Recently, macrolides have been reported to possess wider biological activities such as antiinflammatory,[2,3] antitumor,[4] and antimalarial[5] properties. They are also often used against respiratory tract infections by *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*.[6,7] Macrolide antibiotics inhibit bacterial protein synthesis by binding to the bacterial ribosome, blocking the exit tunnel through which new polypeptides leave. Peptide synthesis is typically halted at the tetrapeptide or pentapeptide stage.[8] We have previously used NMR spectroscopy to characterize the interactions of macrolide antibiotics with bacterial ribosomes and identified structural elements responsible for binding.[9−11] The increasing resistance to antibiotics has become a global problem, and much effort is now directed toward new and more potent classes of drugs to fight the resistance mechanisms. A crucial step in the discovery of novel compounds for preventing resistance is to understand how macrolides interact with their target ribosome and bacterial membranes. A reason for the success of macrolides as drug molecules are also their favorable physicochemical properties and high accumulation in cells and tissues.[12] It is clear that in order to understand their overall biological properties, knowledge of macrolide−membrane interactions is important. Furthermore, it has been reported that macrolides accumulate in lysosomal membranes and induce phospholipidosis as a side effect by inhibiting phospholipase A_1 activity.[13,14] Phospholipidosis is a lysosomal storage disorder characterized by the excess accumulation of phospholipids in tissues.[15,16] The onset of phospholipidosis in preclinical testing in animals can delay or even prevent the drug development process.[14] Some recent results supported observations that macrolides accumulate within cells due to their sequestration in lysosomes.[12] Furthermore, it was also shown that dicationic macrolide antibiotics markedly inhibit endocytosis by binding to the lipids and thereby reducing their mobility. Here we report on interactions of selected macrolide compounds (azithromycin, erythromycin A, azahomoerythromycin, decladinosylazithromycin, clarithromycin, azithromycin aglycone, 3′-*des*(dimethylamino)-clarithromycin, and 3′-*des*(dimethylamino)-3′,4′-didehydro-clarithromycin) (for structures, see the Supporting Information (SI)) with membrane-mimetics. For our studies, we used dodecylphosphocholine (DPC) and sodium dodecylsulphate (SDS) micelles to investigate the binding of macrolide antibiotics using self-diffusion NMR experiments. The orientation and location of the drugs in the membrane-mimetic was determined using relaxation enhancements in the presence of a freely soluble and inert paramagnetic agent as previously demonstrated for membrane-bound peptides.[17−20] Furthermore, possible interactions of macrolides with phospholipase A_1 and phosphatidylserine were investigated.

RESULTS AND DISCUSSION

Binding of Macrolides to Membrane-Mimetics. To obtain information about the mode of interaction of macrolide antibiotics with membrane-mimetics, we carried out NMR self-diffusion and solvent paramagnetic relaxation enhancement experiments of the macrolides azithromycin, erythromycin A,

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azahomoerythromycin, clarithromycin, decladinosylazithromycin, and azithromycin aglycone. The uncharged macrolides 3′-*des*(dimethylamino)-clarithromycin and 3′-*des*(dimethylamino)-3′,4′-didehydro-clarithromycin are basically insoluble in water and also did not dissolve in DPC or SDS solutions. This shows that not only for solubility in water but also for membrane-binding the positively charged amino groups are essential. Because of the insolubility of these two compounds, no further experiments could be carried out with them. For all other compounds, self-diffusion coefficients were obtained for free macrolides and in the presence of deuterated DPC or SDS. DPC and SDS are most commonly used as membrane-mimetics for solution NMR studies because they are readily available in deuterated form, and the resulting micelles are small enough to prevent extensive line broadening in NMR spectra.[21,22] Upon the addition of either SDS or DPC, all soluble macrolides tested showed a significant reduction in self-diffusion coefficients indicative of binding to the micelles (Figure 1).

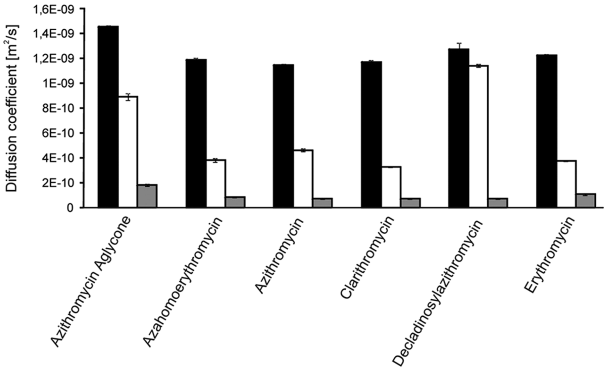

Figure 1. Self-diffusion coefficients (in $m^2/s \times 10^{10}$) of free macrolides (black) and in the presence of DPC (white) or SDS (gray).

Binding to negatively charged SDS is generally stronger than to zwitterionic DPC. Azithromycin aglycone binds significantly weaker than all other macrolides to DPC micelles and is also the weakest binder to SDS. These data indicate that an interaction between the positive charges of the amino groups on the macrolide and the desosamine ring are most important for micelle binding. This electrostatic interaction is somewhat loosened in zwitterionic membranes. This is also in accordance with the observation that phospholipidosis is mainly observed in negatively charged lysosomal membranes where macrolide antibiotics accumulate. In addition, the cladinose ring also strengthens this interaction at least with zwitterionic membrane-mimetics. Using the experimental diffusion coefficients of free macrolides and the ones in the presence of micelles, we calculated mole fraction partition coefficients between macrolide antibiotics and the membrane-mimetic, which are given in the SI. For all macrolides tested, even azithromycin aglycone, which shows no antimicrobial activity, they show tight binding to SDS and for all but azithromycin aglycone and decladinosylazithromycin also to DPC micelles. The binding to membranes is obviously unrelated to the binding to ribosomes and its antimicrobial activity, which has not been observed for the azithromycin aglycone. Information about the orientation and localization of the macrolides in the membrane-mimetic was obtained by titrating the soluble and inert paramagnetic agent Gd(DTPA-BMA) into the solutions of macrolides bound to DPC micelles. By adding this compound to the solvent, the environment around the micelle is made paramagnetic. This leads to solvent PREs (paramagnetic relaxation enhancements) which depend on the distance to the surface of the micelle and allow the positioning of individual nuclei within the micelle.[18−20,23] Gd(DTPA-BMA) has been shown to be inert against proteins and micelle forming lipids.[20,24] Experimental solvent PREs for the investigated macrolides are indicated on the prototypic structure of azithromycin in Figure 2.

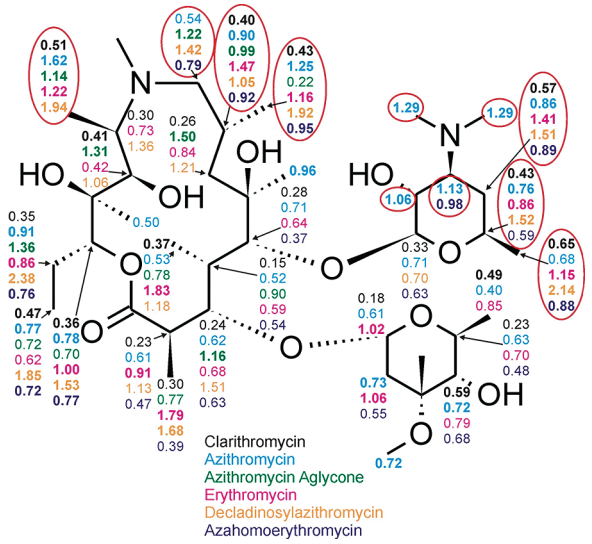

Figure 2. Solvent PREs of all soluble macrolides are drawn at the corresponding position of the prototypical macrolide frame of azithromycin. The values for the individual compounds are color-coded as indicated below. For each macrolide, the higher half of solvent PREs are indicated by bold letters. Protons for which high PREs are found for the majority of compounds are encircled in red.

Because of different binding strengths, the absolute values cannot be directly compared. Therefore, the PREs were grouped for each macrolide separately into high (bold) and low (regular font) values. High values indicate binding closer to the micelle surface. Protons for which the majority of macrolides showed high PREs are encircled in red. They all belong to resonances close to the positively charged amino groups, which therefore have to be closest to the surface of the micelle. As an example, a picture of the orientation of azithromycin in DPC micelles, based on the experimental solvent PREs, is shown in Figure 3 using an energy minimized structure of this macrolide antibiotic. This topology is in good agreement with the orientation of the same compound in lipid bicelles.[25] Therefore, micelles are obviously sufficiently large

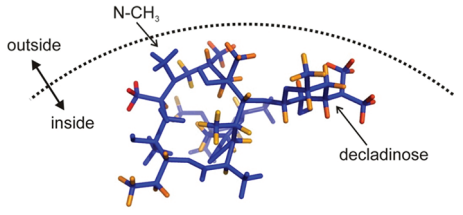

Figure 3. Three-dimensional representation of the orientation of azithromycin in DPC micelles showing the protons for which solvent PREs could be obtained color-coded from red (high) to yellow (low).

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membrane mimetics for the comparatively small macrolide molecules. Another previous study by fluorescence spectroscopy also indicated binding of azithromycin close to the interfacial region of membrane-mimetics.[26] All macrolides tested show roughly the same orientation with the desosamine (prime letters) unit being closer to the surface than the cladinose (double prime letters) sugar unit and the macrolide penetrating with protons on carbons 2, 3, and 4 furthest into the micelle and having 8, 9, and 10 closer to the surface. This orientation allows electrostatic interaction between the positively charged amino groups of the macrolides with the negatively charged phosphate group of the phospholipid headgroup.

The absolute size of the solvent PREs (~0.3−1.2 s^{-1} mM^{-1}) indicates that the macrolides are bound close to the surface. A location near the polar headgroups of the lipids also makes sense due to the large number of hydroxyl and charged amino groups and the assumed electrostatic interaction between the macrolide amino group(s) and the negative charges on the membrane-mimetic.

Azithromycin−Phosphatidylserine−Phospholipase A1 Interactions. To investigate the role of macrolides in phospholipidosis, we studied the interaction of phospholipase A1 with phosphatidylserine in the absence and presence of azithromycin by one-dimensional NMR spectroscopy. The 1D ^{1}H spectrum of the lipid shows only rather weak and broad signals indicative of the formation of large micelles (Figure 4).

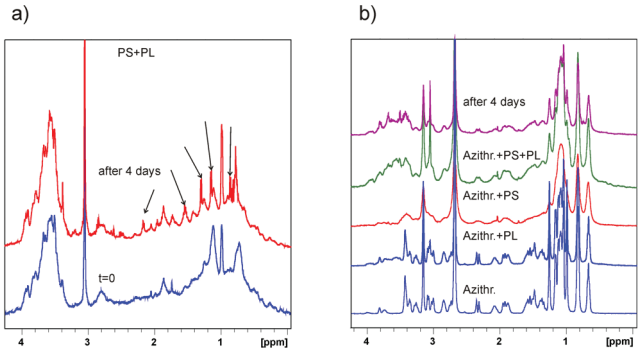

Figure 4. One-dimensional ^{1}H NMR spectra of (a) phosphatidylserine (PS) incubated with phospholipase A_1 (PL) freshly prepared and after 4 days. A spectrum of azithromycin free and mixed with PS and/or PL is shown in (b). While additional sharp signals, indicative of lipid degradation are visible in (a) (indicated by arrows), the presence of azithromycin prevents lipid degradation after 4 days.

The addition of phospholipase A1 did not immediately show any changes in the spectrum of the lipid. However, after 4 days, extensive degradation of the lipid can be observed, as evidenced by the appearance of sharp signals in the ^{1}H NMR spectrum (Figure 4a). The degradation products of phosphatidylserine were analyzed by LC-MS and identified to be lyso-PS (18:0) with a molecular weight of 522 g/mol and 18:1 fatty acid at 281 g/mol (see SI). The sharp signals of azithromycin in aqueous solution are broadened extensively upon the addition of lipid (Figure 4b), indicative of binding.

In the presence of azithromycin, no degradation of phosphatidylserine by phospholipase A_1 can be seen even after 4 days. This protection is however not achieved by binding of azithromycin to phospholipase because the addition of phospholipase to azithromycin does not lead to any changes in the NMR spectrum (Figure 4b). The binding of macrolides to membrane mimetics is close to the surface as found by solvent PREs. Therefore, they obviously block the lipid headgroups from access to phospholipase A1 but not through direct enzyme inhibition (Figure 5).

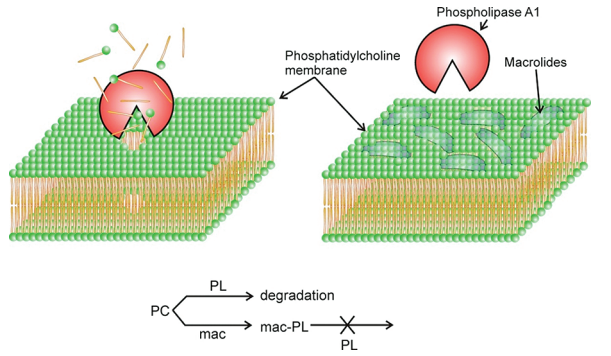

Figure 5. Proposed mechanism of the protecting role of macrolides on lipid membranes. Instead of a direct binding to phospholipase A_1, the macrolides accumulate close to the surface of the membrane and thereby prevent access of the enzyme. PC = phosphatidylcholine, PL = phospholipase A_1, mac = macrolide.

This mechanism is reminiscent of the carpet mechanism of membrane-bound antimicrobial peptides, which kill bacteria by blocking their membranes by binding close to the surface. The resulting indirect inhibition of phospholipase A1 activity results in a higher concentration of nondegraded lipid, which is then accumulated intracellularly and forms lamellated membranous inclusions. It has been discussed previously[14] whether the reduced activity of phospholipase A_1 by cationic amphiphilic drugs is a result of direct enzyme inhibition[27] by the drug or through interactions with the phospholipid.[28,29] Our data show that there is no direct binding of the drugs to phospholipase but rather a protection of the membrane. It might seem surprising that macrolides are protecting the membrane because rather large amounts of the drug are needed. However, it has been shown previously that in lamellar bodies the inducing drug reaches millimolar concentrations[30] and phospholipidosis is only observed when large amounts of cationic amphiphilic drugs are administered.[14] The surface covering of biological membranes by macrolides is likely also involved in the previously described inhibition of endocytosis by macrolide antibiotics. Binding of macrolides close to the membrane surface also does not allow them to penetrate and lyze the membranes, which is in accordance with the absence of the hemolysis (see SI).

CONCLUSION

In conclusion, we were able to prove that protonated nitrogen atoms on macrolides play a crucial role in binding to membranes. They are inserted rather shallow in the membrane with the charged amino groups on the macrolide ring and one sugar unit being close to the polar lipid headgroups. There is no direct interaction between macrolides and phospholipase A_1, but phospholipidosis is instead caused by the protection of lipids by macrolides, obviously by covering the membrane surface.

EXPERIMENTAL SECTION

Materials. Deuterated DPC and SDS were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Gd(DTPA-BMA) was purified from the commercially available MRI contrast agent Omniscan (Nycomed, Oslo, Norway) as described previously.[19]

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All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) in the highest purity available and used without further purification except phospholipase A1 from *Thermomyces lanuginosus*, which was dialyzed against NMR buffer (100 mM KPi pH 6.5, 100 mM NaCl in 90% H_2O/10% D_2O) to remove small molecule additives from the storage solution.

NMR Spectroscopy. NMR spectra to determine solvent PREs were acquired on a Bruker Avance III 700 MHz NMR spectrometer using a 5 mm TCI (HCN) cryo probe. All other NMR spectra were measured on a Bruker Avance III 500 MHz spectrometer using a TXI triple-resonance probe. Self-diffusion coefficients of free macrolides at a concentration of 2 mM, as well as in the presence of 125 mM DPC-d_{38} or 125 mM SDS-d_{25}, were obtained by PGSE (pulsed gradient stimulated echo) spectra as reported previously.[21] Self-diffusion coefficients of free macrolides and in the presence of the membrane-mimetics were used to determine mole fraction partition coefficients as described in the SI. To obtain solvent paramagnetic relaxation enhancements (solvent PREs), the samples were titrated with Gd(DTPA-BMA) as described previously.[19] Interactions within the ternary system macrolide−lipid−phospholipase A_1 were carried out by mixing 2 mM macrolide, 2 mg phosphatidylserine, and 2 mM phospholipase A_1 in all possible combinations. The degradation of lipid was monitored by recording the equivalent spectra after 4 days of incubation at room temperature.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra of aspartate in the absence and presence of phosphatidylserine, results and experimental details of the hemolysis, LC-MS and mole fraction partition coefficients. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DPC, dodecyl phosphocholine; Gd(DTPA-BMA), gadolinium-diethylenetriamine pentaacetic acid-bismethylamide; K_p, mole fraction partition coefficient; Mac, macrolide; PC, phosphatidylcholine; PL, phospholipase; PS, phosphatidylserine; PRE, paramagnetic relaxation enhancement

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