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Membrane interactions of non-membrane targeting antibiotics: The case of aminoglycosides, macrolides, and fluoroquinolones

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ABSTRACT

Numerous antibiotics are known to target intracellular pathways, such as protein translation or DNA replication. Membrane transporters typically regulate drug uptake; however, little is known about direct interactions between these antibiotics and the cell membranes. Here, we studied the interactions between different aminoglycosides (kanamycin, gentamicin, streptomycin, neomycin), macrolides (azithromycin, clarithromycin, erythromycin), and fluoroquinolones (ciprofloxacin, levofloxacin) with bacterial membrane mimics to determine drug partitioning and potential drug-induced membrane disruption. The antibiotics' exact location in the bilayers and their effect on membrane thickness and fluidity were determined from high-resolution X-ray diffraction. While the antibiotics did not change membrane thickness at low (1:100 drug/lipid) or high (1:10 drug/lipid) concentrations, they were found to increase membrane disorder in a dose-dependent manner. However, no membrane damage, such as membrane disruption or pore formation, was observed for any of the antibiotics. To note, all antibiotics partitioned into the lipid head groups, while macrolides and fluoroquinolones also partitioned into the bilayer core. The results suggest that the bacterial membrane is relatively inert in the direct mechanisms of actions of these antibiotics.

1. Introduction

With the worldwide rise of antibiotic resistance, the design and modification of current antibiotics is of critical importance [1,2]. The majority of antibiotics target intracellular biochemical pathways in bacteria. For example, aminoglycosides prevent protein translation, macrolides inhibit protein synthesis, and fluoroquinolones target DNA replication [3]. Aminoglycoside antibiotics are used to treat a wide variety of infections, and mainly target Gram-negative bacteria [4]. Aminoglycosides are recognized to exert their antibacterial action by interacting with the ribosome and interfering with protein translation, thus halting protein synthesis while leaving the cell membrane intact [5]. The mechanism behind the bacterial uptake of this class of antibiotics is a topic of discussion. For instance, it has been proposed that it needs a functional electron transport system, following an energy-dependent process [6], as well as that it penetrates the membrane through porin channels, rather than through direct diffusion [7]. Currently, there is no conclusive mechanism for the uptake of aminoglycosides.

Another family of antibiotics are the macrolides, which are among one of the most used to treat infections caused by Gram-positive bacteria. They are also known to bind to the ribosome and interfere with protein synthesis [8] but their mechanism of entry has yet to be established. However, there is increasing data supporting the idea of an active transport system [9–11].

Fluoroquinolones are an expanding class of broad-spectrum antibiotics used frequently in the treatment of ocular infections [12]. They are known to inhibit enzymes involved in DNA synthesis, more specifically targeting DNA replication [13]. It has been proposed that fluoroquinolones translocate across the membrane through active transport [14]. However, Cramariuc et al. [15] have also suggested that fluoroquinolones simply diffuse through the membrane. Also here, a definitive mechanism seems to be lacking with regards to the transport of these types of antibiotics which is pivotal for determining the inhibitory concentration inside the bacteria.

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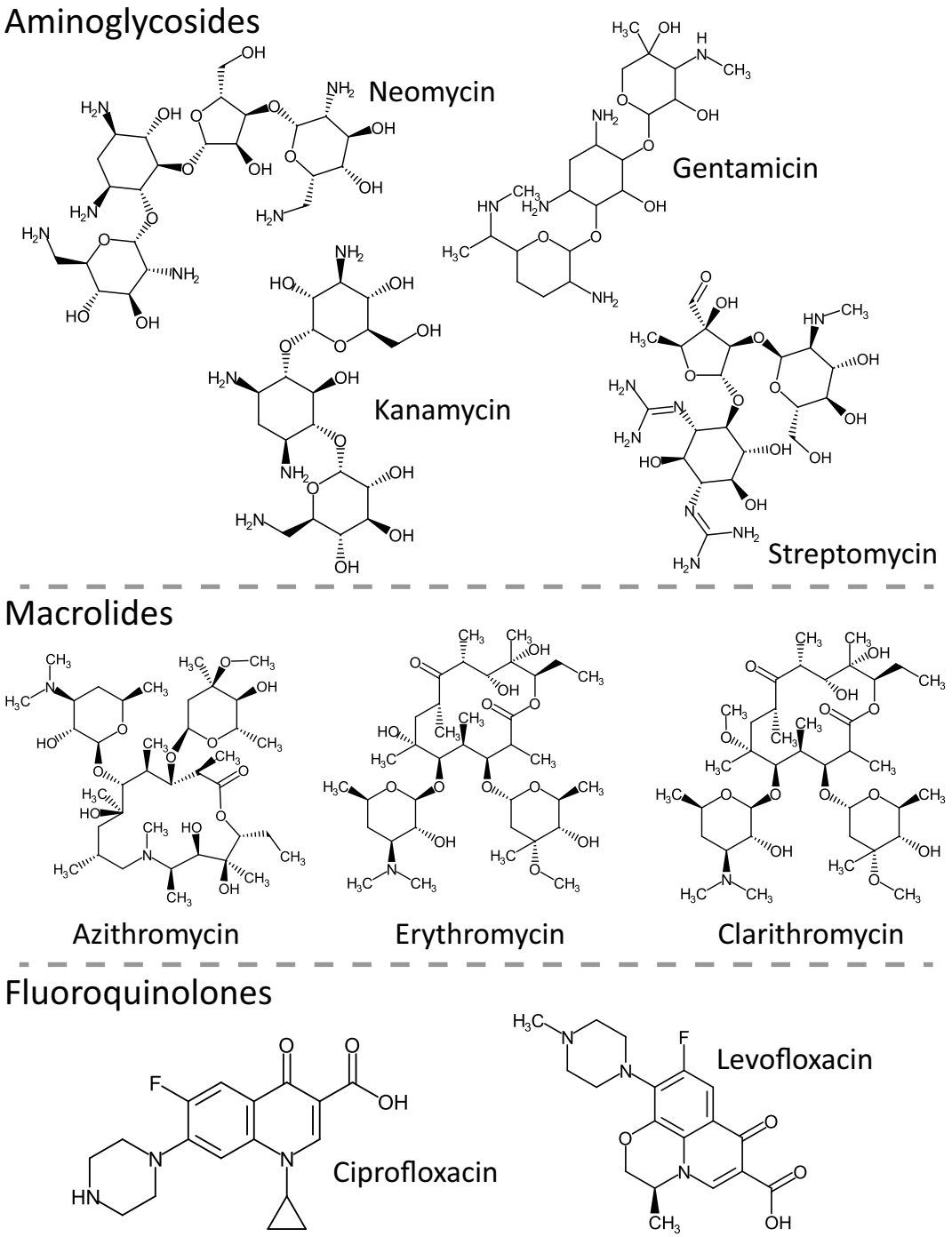

Fig. 1. Molecular structure of the aminoglycosides (kanamycin, gentamicin, streptomycin, neomycin), macrolides (azithromycin, clarithromycin, erythromycin), and fluoroquinolones (ciprofloxacin, levofloxacin) used in this study.

While these drugs have been shown to translocate across the bacterial membranes to reach their respective drug targets [16,17], the lipid bilayer is usually considered an inert interface in the mechanisms of actions for these antibiotics. However, experiments and simulations have for instance shown that Kanamycin A induces membrane disorder; macrolides were found to alter membrane fluidity, and fluoroquinolones were able to partition into the lipid tails [15,18,19]. Moreover, the efficacy of carbapenems has been found to be correlated to their membrane affinities [20]. The understanding of the molecular mode-of-action of non-membrane targeting antibiotics is, therefore, of timely importance in modern drug design.

Here, we studied the interactions between different aminoglycosides, macrolides, and fluoroquinolones with bacterial membrane mimetics *via* biophysical techniques to study whether antibiotics without clear membrane-targeting ability show significant antibiotic-membrane interactions. All molecules are depicted in Fig. 1. Bacterial membrane mimetics were prepared with 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG), and cardiolipin in a 75:20:5 ratio (mol%) [21]. We then prepared drug/lipid ratios of 1:100 and 1:10 with the following antibiotics: kanamycin, gentamicin, neomycin, streptomycin, azithromycin, clarithromycin, erythromycin, ciprofloxacin, levofloxacin. Polymyxin B was included in the study as an antibiotic with strong membrane interaction which leads to significant membrane

damage and pore formation [22,23]. The solutions were deposited on silicon wafers and incubated in humid conditions to create supported lipid bilayers using previously reported protocols [20,22,23]. Small and wide angle X-ray diffraction was conducted to determine the exact position of the antibiotic molecules within the membranes.

2. Materials & methods

2.1. Membrane preparation

Bacterial membrane mimetics were prepared on single-side polished silicon wafers [20,22,23]. 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE, Avanti), 1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG, Avanti), and cardiolipin (Avanti) were mixed in a 75:20:5 ratio (mol%) in 2,2,2-trifluoroethanol:chloroform (1:1, vol/vol) mixture at a solution concentration of 18 mg/mL. We then prepared drug/lipid ratios of 1:100 and 1:10 with the following antibiotics: kanamycin, gentamicin, neomycin, streptomycin, azithromycin, clarithromycin, erythromycin, ciprofloxacin, levofloxacin, and polymyxin B (Sigma Aldrich). Silicon wafers were sonicated in 1,2-dichloromethane for 30 min, and then rinsed with alternating methanol and 18.2 MΩ·cm water. The wafers were dried, and 75 μL of solution was deposited. After drying, the samples were placed in a vacuum for 24 h at 37 °C to allow for trace solvent evaporation and annealing. Samples were then hydrated and incubated in a closed chamber at 98% RH with a separate K_2SO_4 saturated solution for 48 h prior to scanning.

In order to quantify drug-induced structural changes in membrane properties, a drug/lipid ratio of 1/100 and 1/10 was used, which is generally higher than typical antibiotic MIC. To calculate the MIC for a bacterial membrane, we assume a bacterium to have a volume of 7×10^{-13} ml. We multiply the volume by the MIC of the studied antibiotics, such as 0.5 μg/ml for ciprofloxacin [24]. Dividing by the antibiotic's molar mass, 330 g/mol for ciprofloxacin, and multiplying by Avogadro's constant, we obtain 640 drug molecules per cell. Given that the number of bacterial lipids is reported to be 5.4×10^5 [25], the MIC would therefore be 1.2×10^{-1} mol% or roughly 10-fold smaller than we report in 1/100 samples in the case of ciprofloxacin. The drug concentrations in this study are, therefore, above physiological conditions.

2.2. X-ray diffraction

X-ray diffraction data was obtained using the Biological Large Angle Diffraction Experiment (BLADE) at McMaster University. BLADE uses a 9 kW (45 kV, 200 mA) CuKα rotating anode at a wavelength of 1.5418 Å using a Rigaku HyPix-3000 2D semiconductor detector with an area of 3000 mm^2 and 100 μm pixel size [20]. Diffraction measurements were conducted under controlled temperature and humidity conditions (T = 30 °C, 98%RH) in a custom-built humidity chamber. All samples were prepared and measured in replicates to check for consistency. The data presented in this work are the result of individual diffraction experiments conducted on individual membrane samples. Several samples were prepared for each drug and preparation protocols have been refined until the experimental results between different samples were consistent. The good statistics in diffraction experiments, in general, are the result of the large spatial and temporal averages, which include large ensembles and a large number of molecules.

2.3. Out-of-plane structure and electron densities

The electron density was determined through Fourier transform from the out-of-plane diffraction. The relative electron density, ρ(z), can be approximated by a 1-dimensional Fourier analysis,

$$\rho(z) = \frac{2}{d_z}\sum_{n=1}^{N}\sqrt{I_n q_n}\,\nu_n \cos\left(\frac{2\pi n z}{d_z}\right), \tag{1}$$

where N is the highest order of the Bragg peaks observed in the experiment. The integrated peak intensities, I_n, are multiplied by q_n to receive the form factors, $F(q_n)$ [26,27]. The bilayer form factor $F(q_z)$, which is in general a complex quantity, is real-valued in the case of centro-symmetry. The phase problem of crystallography, therefore, simplifies to the sign problem $F(q_z) = \pm |F(q_z)|$ and the phases, ν_n, can only take the values ± 1. The phases ν_n are needed to reconstruct the electron density profile from the scattering data following Eq. (1). When the membrane form factor, $F(q_z)$ is measured at several q_z values in a continuous fashion, $T(q_z)$, which is proportional to $F(q_z)$, can be fit to the data:

$$T(q_z) = \Sigma_n \sqrt{I_n q_n}\, sinc\left(\frac{1}{2} d_z q_z - \pi n\right). \tag{2}$$

In order to determine the phases quantitatively, the form factor has to be measured at different q_z values using the so-called swelling technique or by measuring the bilayer at different contrast conditions when using neutron diffraction. In this work, the phases, ν_n, were assessed by fitting experimental peak intensities and comparing them to the analytical expression for $T(q_z)$ in the above equation. An array of phases $[-1-11-1-1]$ was used for all samples.

ρ(z) is initially calculated on an arbitrary scale, they are then scaled based on the protocol established in our previous work [28]. The curves are scaled until the total number of electrons within the lipid unit cell across a membrane leaflet, $e^- = A_L \int_0^{d_z/2} \rho(z) dz$ agrees with the total number of electrons expected based on the membrane composition. ρ(z) was scaled by the number of electrons per unit cell, including the electron contributions from the membrane lipids, antibiotic molecules, and water, while the bilayer core, z = 0 Å was fixed at 0.22 $e^-/Å^3$ to represent terminal methyl groups of the lipid acyl chain.

ρ(z) shows an increased electron density around $|z| \sim 20$ Å, corresponding to the electron-rich head groups of the lipid molecules, and a decreased density in the center of the bilayer (z = 0 Å). The membrane thickness was determined by the distance between the two maxima in the electron density profile, and will be referred to as head-to-head distance d_{HH}. To determine the degree of orientation of the membranes in the stack, the correlation peak intensities were integrated as function of the meridional angle φ (the angle relative to the q_z axis) as depicted in Fig. 2a). The corresponding intensity was fit with a Gaussian distribution centered at 0, which was then used to calculate the degree of orientation using Hermans orientation function:

$$H = \frac{3 <\cos^2\varphi> - 1}{2}. \tag{3}$$

A small width in the angular distribution is indicative of well ordered membranes within the stack. A degree of orientation between ~82% and ~97% was determined, in good agreement with previous studies on mono- or multi-component synthetic membranes [28]. The experimental errors were determined from fitting errors, in either determining membrane width from linearly fitting peak positions along q_z or Hermans orientation directly from fitting the decay in angular reflectivity. Electron densities were validated from good agreement in $T(q_z)$.

The experimental errors were determined as follows: Errors for peak positions, peak width and peak height are determined as the fit standard errors. Because the pure, undisturbed bacterial membrane mimic is used as a reference when determining the drugs' electron density in Fig. 2, this approach assumes that the changes to the membrane induced by the presence of the drug are small.

3. Results

The 2-dimensional X-ray intensity map for the bacterial membrane-

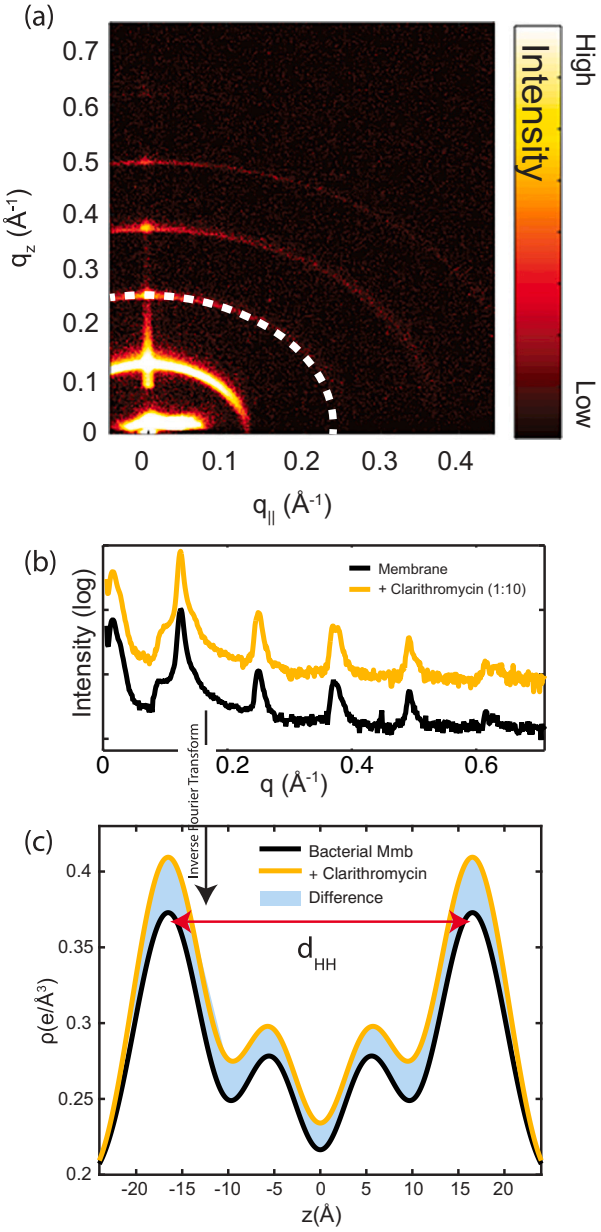

Fig. 2. (a) 2-dimensional X-ray intensity map of the bacterial membrane mimetic with out-of-plane (q_z) and in-plane ($q_{||}$) features. Peak intensity was integrated along the dotted line to determine membrane orientation using Hermans orientation function. (b) A single series of well-defined Bragg peaks along q_z (*i.e.* at 0.12, 0.24, 0.36, 0.48, 0.60 Å^{-1}) is the result of membrane stacking. (c) The integrated intensities of these peaks were Fourier transformed to calculate the electron density, ρ(*z*), normal to the membranes, using claithromycin as an example. The difference in ρ between the pure membrane and the membrane containing the drug was used to determined the position of the drug in the membrane. All electron densities were generated for for high (1:10) drug/lipid concentration to minimize the impact of the drug on the membrane's structure and avoid potential drug-drug interactions.

mimic is shown in Fig. 2(a). The diffraction peaks along the membrane normal (q_z, shown in Fig. 2(b)) are the result of membrane stacking, and were used to determine the membrane width in the absence and presence of each drug. The spacing between each respective peak along q_z, Δq_z, corresponds to the lamellar spacing, which includes the membrane width and thickness of the water layer (~19 Å). The electron density along the membrane normal was determined through Fourier transform and is shown in Fig. 2(c), using claithromycin as an example. The membrane width, d_{HH}, is defined as the distance between the head groups in the electron density, as indicated in the figure. Membrane orientation was determined from Hermans orientation function, *H*, by integrating the peak intensity along the dotted line in Fig. 2(a). $H = 1.0$ corresponds to lipids which are perfectly parallel to each other within the bilayer (hyper-ordered), whereas $H = 0.25$ corresponds to a membrane with lipids in complete disorder.

Membrane width and orientation for all antibiotics are shown in Fig. 3. In Fig. 3(a), the low and high concentrations of the different antibiotics showed no significant difference in membrane width, suggesting that all studied antibiotics do not impose a compressive force on the bilayer within the experimental errors. Membrane orientation for all compounds is shown in Fig. 3(b). For reference, a normal eukaryotic model membrane shows $H = 0.90$ [28]. Notably, kanamycin and clarithromycin reduced membrane order at low concentrations. The antibiotics showed a concentration-dependent effect: At high concentrations of each antibiotic, the membranes were found to be significantly disordered from their native state. Polymyxin B was included as reference for a drug that strongly interacts with bacterial membranes and leads to membrane damage and pore formation. While all *H* values for the drugs in this study at high concentrations show values of ~0.7, they induce significantly less membrane disorder than polymyxin B with values of $H \sim 0.55$ and are, therefore, above the threshold of inducing membrane damage or pore formation.

The electron density ρ(*z*) was calculated in the absence and presence of all drugs; the results are shown in Fig. 4. An increased density between 15 Å and 20 Å generally corresponds to a drug which prefers to localize within the membrane head groups, whereas a position at less than 15 Å indicates drug partitioning into the hydrophobic lipid tails. All antibiotics and bacteria membrane mimic systems show an increased electron density in the head group and tail region compared to the bacteria membrane mimic alone. This indicates that aminoglycosides, macrolides and fluroquinolones partition into the bacterial membrane. Furthermore, antibiotics from the macrolide and fluroquinolones are also seen to partition into the bilayer core, leading to an increase in the electron density in the center of the membrane We note that these findings are in good agreement with previous theoretical studies with aminoglycosides and fluoroquinolones [15,18] and macrolides [29].

4. Discussion

While antibiotics with intracellular targets typically translocate across the bacterial membranes through active transport, it was suggested that certain antibiotics can diffuse through the membranes passively. As a first step, these drug molecules need to spontaneously partition in the bilayers, preferably in the hydrophobic membrane core to eventually enable transmembrane diffusion. An important question with clinical relevance is if partitioning of the antibiotics leads to changes in membrane properties and potentially to membrane damage or rupture. The concentration of drugs used in this work are 10-fold higher than typical MIC in clinically relevant cases. Elevated concentrations are typically required for biophysical analyses to make the subtle signals detectable. To note, similar concentrations of carbapenems provided mechanistic insights into action and was corroborated with various bacterial strains [20]. In more physiologically relevant systems, higher concentrations of drugs are indeed possible at collecting sites, such as the kidney or liver [22,30].

All antibiotics studied were found to spontaneously partition in the bacterial membrane mimics to some extent. From measuring membrane width and disorder we find that none of the antibiotics significantly altered membrane width or fluidity. Membrane disorder was induced at high concentrations of the antibiotics, which led to an increase of lipid dynamics and a decrease of the order parameter of the lipids, as previously observed from high concentrations of kanamycin [18]. The antibiotics in this study were not found to induce membrane compression or swelling. By comparing the structural membrane

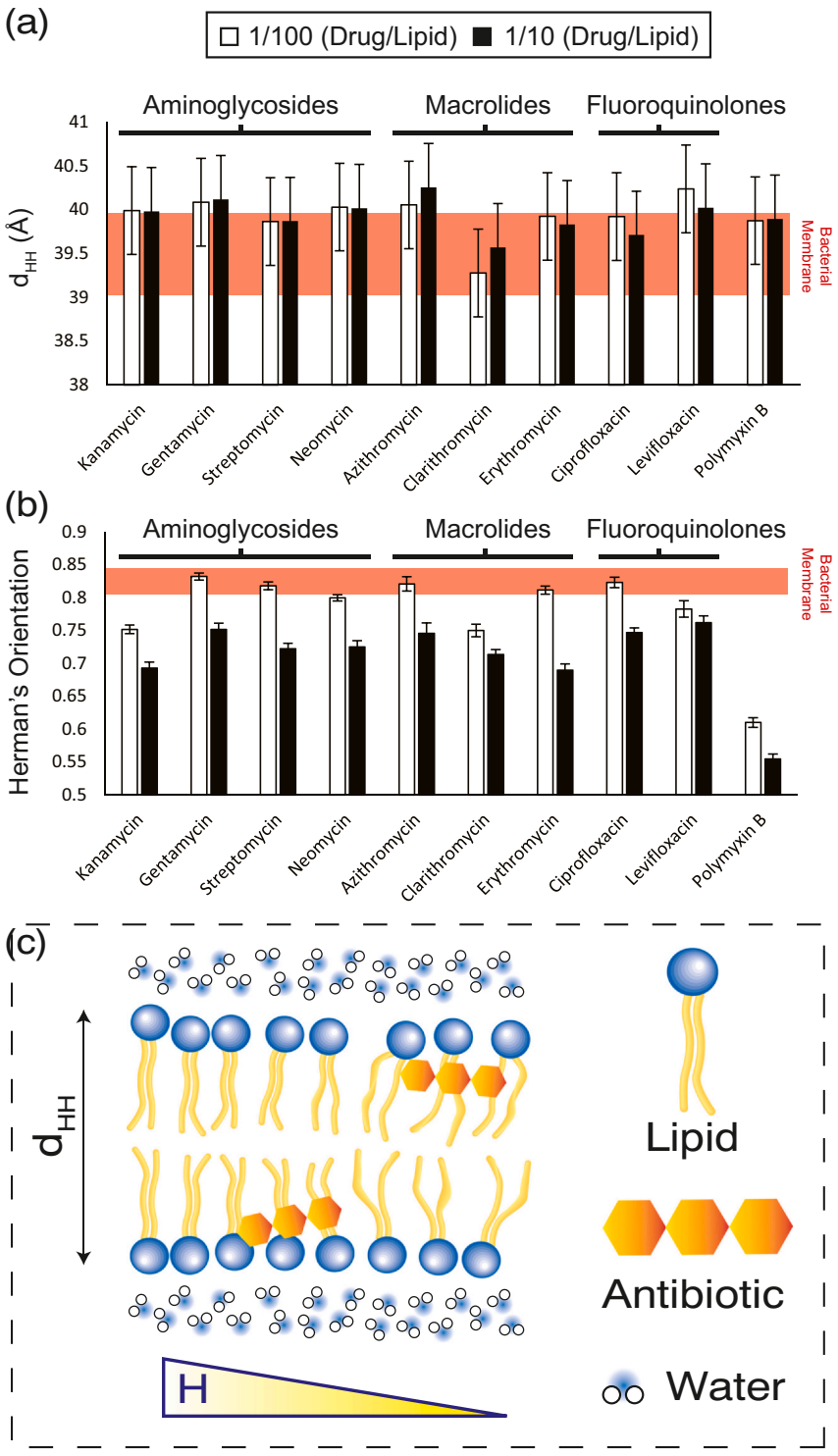

Fig. 3. (a) Membrane width for the pure bacterial membrane mimic (red) and with each antibiotic at low and high antibiotic concentrations. Width was found to be constant within the experimental errors. (b) Hermans orientation for membranes containing the different antibiotics. Smaller H values correspond to increased membrane disorder. While the antibiotics had little effect on the membrane at low concentrations, membrane disorder was found to significantly increase at high concentrations. (c) Cartoon to visualize d_{HH} and H. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

parameters with the effect of polymyxin B, a membrane disrupting antibiotic, the structural changes were much smaller and the observed values were well above the threshold of expected membrane rupture or membrane pore formation. Thus, the non-membrane targeting antibiotics in this study are not expected to significantly damage global membrane properties, even at elevated concentrations.

The mechanism of translocation of the studied drug classes into the bacterial cytoplasm is an important consideration in drug design. The presence of transporters, shuttles, or large barrel channels such as OmpA, MerF, and others play the major role in the translocation of these drugs. We find that the antibiotics in this study were able to spontaneously partition both in the head groups into the lipid tails. Additionally, macrolides and fluoroquinolones show some partitioning into the membrane core. A certain proportion of these two antibiotic classes may therefore be able to translocate the bacterial membrane. Although the passive diffusion of these drugs through membranes likely plays a minor role, it may change the active concentrations of the drugs within the bacteria.

5. Conclusion

The results show that aminoglycosides, macrolides, and

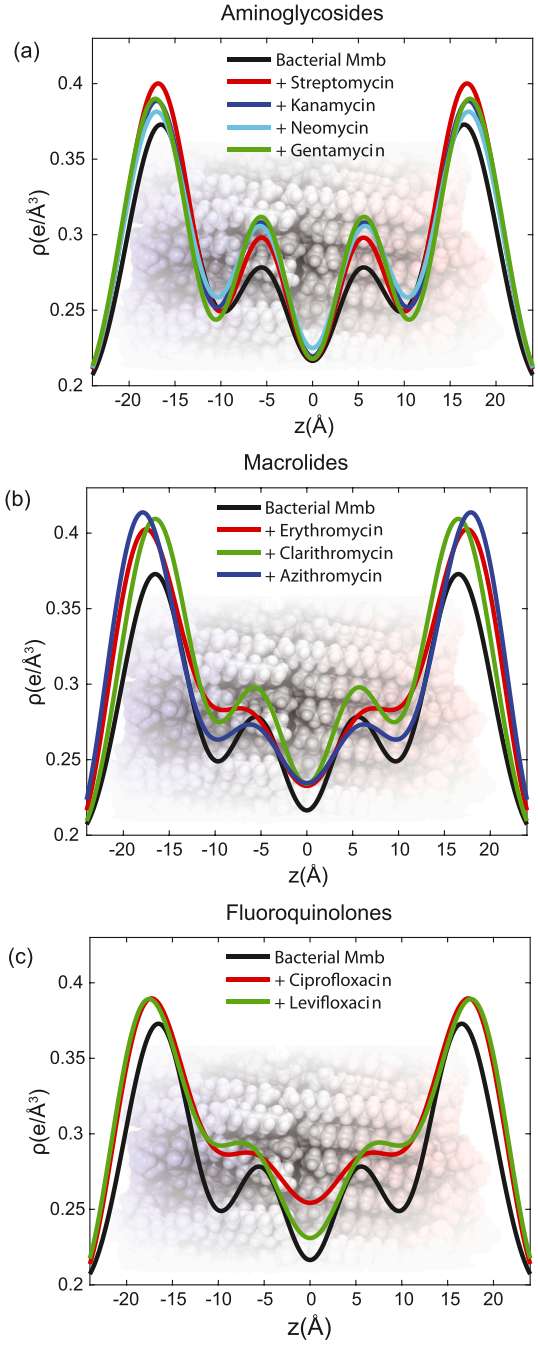

Fig. 4. Electron density profiles for the antibiotic and bacterial membrane mimic systems. Each panel corresponds to a different antibiotic class at 1/10 drug/lipid ratios. The underlying cartoon shows the position of the lipid bilayer relative to the peaks in electron density. The lipid head groups are located at ~19 Å from the bilayer center (0 Å corresponds to the membrane center). All drugs were found to partition in the bacterial membrane mimics preferably in the head group region with some parts of the molecule reaching into the membrane core, as can be seen by the increase in the electron density profile of the antibiotic systems compared to the bacterial membrane alone both in the head group and tail region.

fluoroquinolones spontaneously partition in bacterial membrane mimics. While all studied antibiotic families were found to partition in the lipid head groups and tails, macrolides, and fluoroquinolones were additionally found to partition in the membrane core. No significant change in membrane width or increase in membrane disorder was observed when a drug-membrane complex is formed. Membrane disorder was found to increase for all drugs at high drug concentrations; however, far below the threshold of membrane damage or pore formation. Together, these results support the assumption that the membrane is a relatively inert surface in the mechanisms of actions of these antibiotics.

Declaration of competing interest

None.

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