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Decrease of elastic moduli of DOPC bilayers induced by a macrolide antibiotic, azithromycin

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Abstract

The elastic properties of membrane bilayers are key parameters that control its deformation and can be affected by pharmacological agents. Our previous atomic force microscopy studies revealed that the macrolide antibiotic, azithromycin, leads to erosion of DPPC domains in a fluid DOPC matrix [A. Berquand, M. P. Mingeot-Leclercq, Y. F. Dufrene, Real-time imaging of drug-membrane interactions by atomic force microscopy, Biochim. Biophys. Acta 1664 (2004) 198-205.]. Since this observation could be due to an effect on DOPC cohesion, we investigated the effect of azithromycin on elastic properties of DOPC giant unilamellar vesicles (GUVs). Microcinematographic and morphometric analyses revealed that azithromycin addition enhanced lipid membranes fluctuations, leading to eventual disruption of the largest GUVs. These effects were related to change of elastic moduli of DOPC, quantified by the micropipette aspiration technique. Azithromycin decreased both the bending modulus (k_c, from 23.1±3.5 to 10.6±4.5 k_BT) and the apparent area compressibility modulus (K_{app}, from 176±35 to 113±25 mN/m). These data suggested that insertion of azithromycin into the DOPC bilayer reduced the requirement level of both the energy for thermal fluctuations and the stress to stretch the bilayer. Computer modeling of azithromycin interaction with DOPC bilayer, based on minimal energy, independently predicted that azithromycin (i) inserts at the interface of phospholipid bilayers, (ii) decreases the energy of interaction between DOPC molecules, and (iii) increases the mean surface occupied by each phospholipid molecule. We conclude that azithromycin inserts into the DOPC lipid bilayer, so as to decrease its cohesion and to facilitate the merging of DPPC into the DOPC fluid matrix, as observed by atomic force microscopy. These investigations, based on three complementary approaches, provide the first biophysical evidence for the ability of an amphiphilic antibiotic to alter lipid elastic moduli. This may be an important determinant for drug: lipid interactions and cellular pharmacology.

Keywords: Micropipette; Giant vesicle; Bending modulus; Area compressibility modulus; Azithromycin; DOPC

1. Introduction

Interactions of drugs with biological membranes may account for their activity and/or toxicity, thus represent an important area of investigation. Artificial lipid bilayers are increasingly used as models of cell membranes for biophysical studies of lipid:lipid, lipid:protein, and lipid:drug interactions.

Using atomic force microscopy on supported artificial phospholipid bilayers, we showed that azithromycin, a macrolide dicationic antibiotic [2,3] (Fig. 1), not only leads to the erosion and eventual disappearance of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) gel phase domains surrounded by a fluid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) matrix, but also increases the fluidity at the hydrophilic/hydrophobic interface of DOPC:DPPC [1,4]. To test whether these alterations resulted from changes in membrane cohesion of the bulk lipid phase, we here addressed the effect of azithromycin on the elastic properties of DOPC bilayers.

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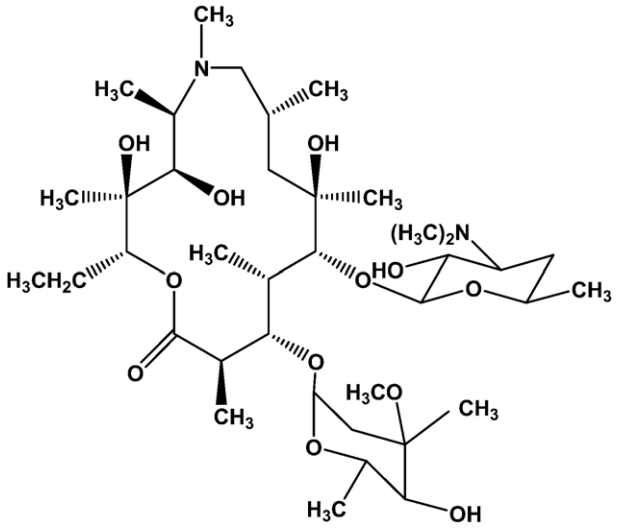

Fig. 1. Structural formula of azithromycin (9-deoxo-9a-methyl-9a-azahomoerythromycin A).

Following the pioneering work of Helfrich [5,6], numerous efforts have been devoted over the last three decades to determine the biophysical parameters of membrane cohesion and elasticity, in particular the bending properties which control bilayer fluctuations and vesicle shape in relation to intrinsic bilayer properties such as adhesion, in- or e-vagination and lipid: protein interactions [7–9]. The two major parameters reflecting the elastic properties of a bilayer are the bending modulus (k_c), reflecting the energy associated to spontaneous thermal fluctuations of the membrane, and the apparent area compressibility modulus (K_{app}), reflecting the energy required to stretch a bilayer.

In present work, we used giant unilamellar vesicles (GUVs) made of DOPC to study the effect of azithromycin on the overall shape of vesicles, and to measure the bending modulus and the apparent area compressibility modulus by the micropipette aspiration technique [10,11]. Results were related to the location of azithromycin at the hydrophilic/hydrophobic interface, the mean surface occupied by each DOPC molecule and the energy of interaction between DOPC molecules as determined by molecular modeling.

2. Materials and methods

2.1. Reagents

DOPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification; Tris (Tris-hydroxymethyl-aminomethane) was from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), glucose and sucrose were from Fluka (Buchs, Switzerland). Azithromycin (potency = 94.4%, MW = 785 g/mol) was supplied as dihydrate free base by Pfizer (Groton, CT, USA). Since azithromycin is sparingly soluble in water at pH 7.0, but very soluble at acidic pH, a stock solution was prepared by dissolving 22.5 mg of the free base in 1 ml 0.1 M HCl (28.6 mM) and further diluted in Tris buffer (10 mM, pH 7.4) or in Milli-Q water just before the experiments, as indicated.

2.2. GUVs preparation

DOPC-GUVs were prepared by electroformation [12]. Ten μl of a DOPC solution (1 mg/ml in $CHCl_3$) were spread onto the Indium Tin Oxide (ITO)-coated side of a glass slide, followed by vacuum drying overnight. A growing chamber for vesicle electroformation was mounted by placing a covering plate over the conductive glass slide containing the lipidic film and by sealing both plates to each other with Vitrex wax (Vitrex, Denmark) with a 1-mm spacer. For direct morphological studies, the lipid film was hydrated with a 100 mM sucrose solution. For micropipette aspiration studies, the lipid film was hydrated with 100 mM sucrose supplemented or not with azithromycin solution (50 μM, final concentration). For both studies, GUVs were grown by applying an alternative voltage (2 V, 10 Hz) across the growing chamber for about 1 h.

2.3. Transfer of GUVs

Giant vesicles were transferred either into an observation chamber or a manipulation chamber filled with 102 mM glucose. The slight density difference between the 100 mM sucrose and 102 mM glucose solutions drived the vesicles toward the bottom plate where they could be easily handled and observed. The glucose and sucrose concentrations osmotically matched the outer and inner solutions (Osmometer Knauer, Osmo 2320, Berlin, Germany), so as to avoid vesicle swelling or shrinkage. A thin glass slide covered the spacer to avoid evaporation. In such conditions, GUVs were stable during at least 1 day [13].

For the observation of GUVs, we used a chamber made of a glass slide with a spacer (CoverWell, Grace Bio-Labs, Bend, OR, USA). To test the effect of azithromycin, the cavity of the chamber was filled with a mixture of 100 μl of GUVs suspension in 102 mM glucose and 30 μl of a solution containing glucose and azithromycin, to reach a final 50 μM azithromycin concentration. Since not all lipid initially deposited on the ITO-coated slide becomes organized in giant vesicles, it was not possible to evaluate either the exact amount of phospholipid transferred in the observation or manipulation chambers, nor the DOPC:azithromycin ratio.

For GUVs handling, we used a manipulation chamber made of a thin glass slide with a spacer (CoverWell). The inner volume of the spacer was around 0.6 ml. Hundred μl of a vesicle suspension were transferred from the growing chamber to the manipulation chamber containing 0.5 ml of a 102 mM glucose solution. To prevent leakage of azithromycin entrapped in GUVs during their preparation, azithromycin was added to the manipulation chamber to a final 25 μM concentration. For micromanipulation of GUVs in presence of azithromycin, the stock antibiotic solution was diluted in water instead of Tris buffer, in order to minimize charges in the manipulation chamber.

2.4. Morphological studies

Several hundreds of GUVs, preincubated or not with 50 μM azithromycin for 30 min, were recorded with an inverted microscope (Axiovert S100, Zeiss, Germany) to determine the vesicle size distribution (Axiovision 4.4 program, Zeiss, Göttingen, Germany). Briefly, after recording grey level images, individual objects were resolved by segmentation (binary images: white GUVs on a black background) and residual open structures were filled. Surfaces of individual GUVs were measured together with a shape factor (circle = 1; line = 0). Only GUVs with a shape factor >0.9 and a surface >8 pixels^2 (5.7 μm^2) were retained for the analysis. Each processed image was inspected one by one to ensure the complete and exclusive selection of GUVs (97% of recorded GUVs satisfied these criteria). The morphometric analysis was performed by an independent investigator, unaware of GUVs treatment.

2.5. Micropipette aspiration of GUVs

The bending modulus and the apparent area compressibility modulus were determined by controlled aspiration of GUVs into micropipettes made by pulling borosilicated capillary glass tubing (Phymep, Paris, France) with a microforge (F-1200, de Fonbrune, Alcatel, France). To avoid charge accumulation on the glass, the micropipette and the glass slide at the bottom of the manipulation chamber were first pretreated with a 0.05% (w/w) BSA solution in water during 10 min, then rinsed with distilled water to remove unbound BSA. The micropipette, with a final diameter of ~10 μm, was fixed on the stage of an inverted microscope and was driven by a *xyz* shift with an AIS2 micromanipulator device (CellBiology Trading, Hamburg, Germany) to contact selected vesicles. Then, a pressure difference between the interior and the exterior of the

micropipette was applied through an hydraulic system equipped with a digital micrometer (DMH-1, Newport Corporation, Irvine, CA, USA), with an accuracy of 10 mPa. Images of GUVs were obtained with a C5405 camera (Hamamatsu, Japan) using a 40× magnification and analyzed with the AIS2 software.

2.6. Determination of the bending modulus (k_c) and the apparent area compressibility modulus (K_{app}).

The relation between the apparent surface change area (α) and the tension of the membrane (σ) was determined using a micropipette apparatus, as first developed by Evans [10,14] to extract both k_c and K_{app} moduli. These biophysical measurements of the membrane mechanical intrinsic properties were used to quantify alterations induced by azithromycin. The α and σ parameters were determined as follows: a cylindrical micropipette of internal radius r held a vesicle with a pressure difference $\Delta P = Pi - Pe$, where Pi and Pe are the inner and outer micropipette pressure, respectively (Fig. 2). Aspiration of the vesicle, originally a sphere of radius R, generated a cylindrical component of length L and a hemispherical cap of radius r. The radius of the remaining vesicle outside of the micropipette decreased to R.

Laplace's law allows one to determine the tension, σ, from these geometrical values:

$$\sigma = \frac{\Delta P}{2}\left(\frac{r}{1-(r/R)}\right) \tag{1}$$

Parameter α is defined as the relative increase of the apparent area of the vesicle, from an initial state $A_{a,0}$, when the vesicle was submitted to some minimal pressure difference ΔP_0, to a final state A_a corresponding to a given pressure difference ΔP:

$$\alpha = \frac{A_a - A_{a,0}}{A_{a,0}} = \frac{1}{2}\frac{r}{R^2}L\left(1-\frac{r}{R}\right) \tag{2}$$

The fundamental constitutive equation of fluid bilayers, established by Helfrich [5], reads:

$$\frac{A - A_a}{A_a} = \frac{k_B T}{8\pi k_c}\ln\frac{\sigma}{\sigma_0} + \frac{K_{app}}{\sigma} \tag{3}$$

where k_B is the Boltzmann constant, T is the absolute temperature in Kelvin degrees, σ_0 is a reference value of the tension for each vesicle, A is the actual area of the lipid vesicle, k_c is the bending modulus and K_{app} is the apparent area compressibility modulus.

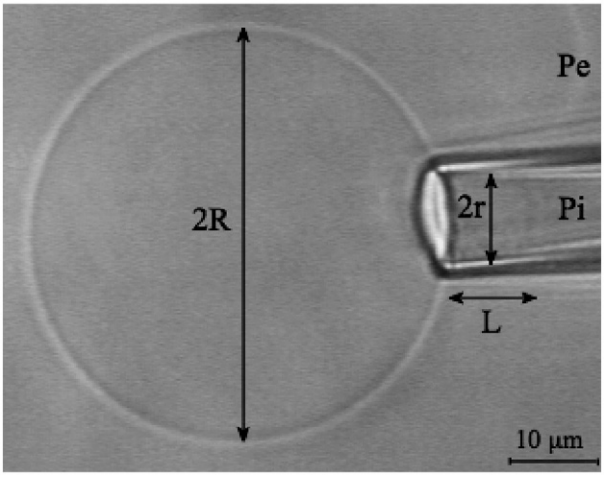

Fig. 2. Typical image of a giant unilamellar vesicle (R=23.8 µm) under contact and minor suction by the micropipette (r=9.6 µm). Tension (σ) was 1.3.10^{-7} N/m and pressure difference ($\Delta P = Pe - Pi$) was 0.3 Pa.

The Helfrich's law (Eq. (3)) then becomes:

$$\alpha = \frac{k_B T}{8\pi k_c}\ln\frac{\sigma}{\sigma_1} + \frac{K_{app}}{\sigma} \tag{4}$$

where σ_1 is the value of the first measured tension. In the low σ regime, some of the real area A was hidden in sub-optical fluctuation modes and only the apparent projected area A_a could be optically measured. Thus, a plot of the natural logarithm of the tension, σ as a function of α for successive values of the pressure differences ΔP, allowed one to derive k_c. In the high σ regime, the bilayer was extended in the lateral dimension, and a plot of σ as a function of α allowed for the calculation of K_{app}.

2.7. Interaction between azithromycin and phospholipids by molecular modeling using the Hypermatrix procedure

The Hypermatrix procedure was used to study the interaction between azithromycin and DOPC molecules, as previously described [15]. In this method, the lipid:water interface is modelised by linearly varying the dielectric constant ε between 3 (above the interface) and 30 (below the interface). The initial position and orientation of azithromycin and the lipids are those defined using the TAMMO procedure, taking into account the hydrophobic and hydrophilic centers of the molecule [15].

The position of azithromycin was kept constant and parallel to Y axis while the first lipid molecule translated towards the azithromycin molecule along the axis by l steps of 0.1 nm. It rotated by steps of 10° around its Z' axis and around the X axis: l is the number of positions tested along the X axis, m is the number of rotations around azithromycin and n is the number of rotations around the lipid itself. For each set of l, m and n values, the energy of interaction between azithromycin and lipid was calculated as the sum of Van der Waals, electrostatic and hydrophobic terms. Then, for each set of values l, m and n, the lipid molecule moved by step of 0.05 nm along the Z' axis perpendicular to the interface and the angle of Z' axis bended by +1.5° with respect to the Z axis. The energy values together with the co-ordinates of all assemblies were stored in a matrix and classified according to decreasing values. The most stable matching was selected to set the position of the first lipid. The position of the second lipid was then defined as the next most energetically favourable orientation stored in the hypermatrix, taking steric and energetic constraints due to the presence of the first lipid molecule into account. To further minimize the energy of the tri-complex, the position of both lipid molecules was alternatively modified according to the energy classification of the hypermatrix. For the next lipid molecule, the same process was iterated but the positions of all surrounding molecules were alternatively modified in order to derive the lowest energy state. In these calculations, the energy of interaction between all lipids is minimized. The process was ended when azithromycin was completely surrounded with lipids.

The azithromycin:DOPC assembly was then inserted into an implicit simplified bilayer using the IMPALA method described previously [16]. This method simulates the insertion of any molecule into a bilayer by adding energy restrain functions to the usual energy description of molecules. The lipid bilayer was defined by $C_{(z)}$, which represents an empirical function describing membrane properties. This function is constant in the membrane plane (X and Y axes) but varies along the bilayer thickness (Z axis) and more specifically, at the lipid: water interface corresponding to the transition between lipid acyl chains (no water=hydrophobic core) and the hydrophilic aqueous environment:

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

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

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

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

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

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

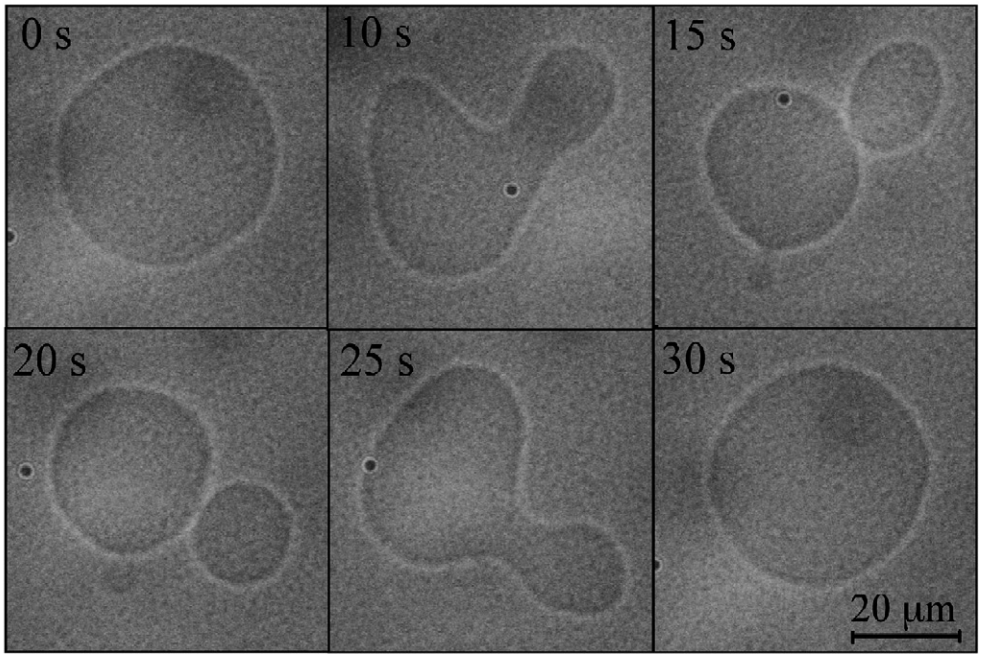

Fig. 3. Qualitative evidence for the destabilization of GUVs by azithromycin. Typical fluctuations of a GUV exposed to 50 μM azithromycin over 30 s. Scale bar=20 μm.

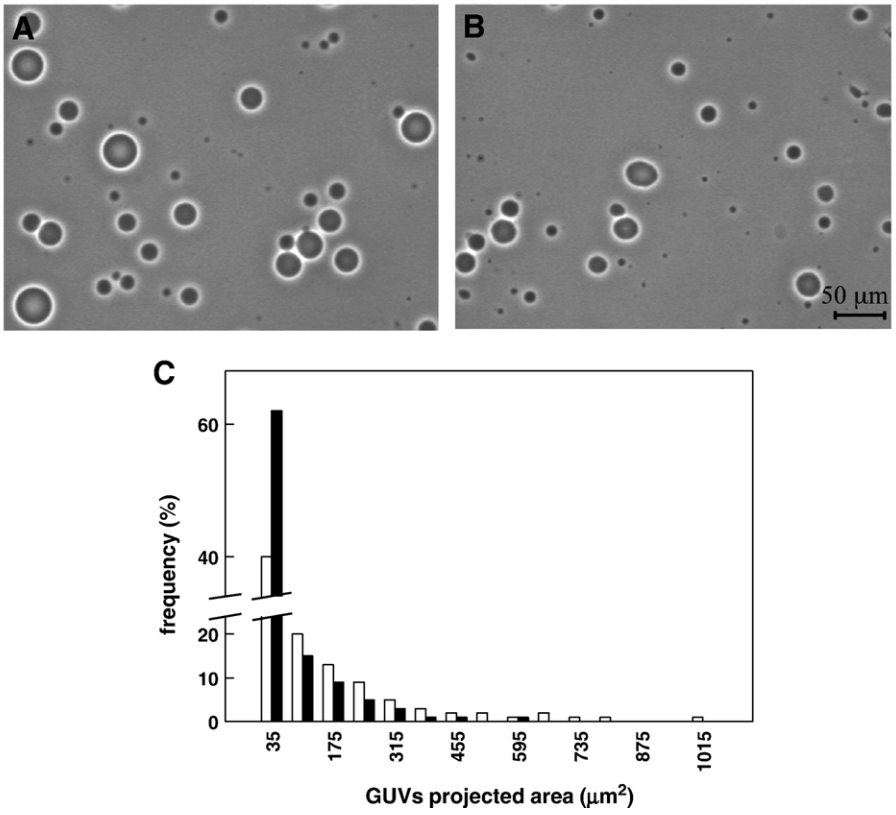

Fig. 4. Effect of azithromycin on the size distribution of GUVs. GUVs were incubated for 30 min in a glucose solution without (A) or with 50 μM azithromycin (B). Scale bar=50 μm. (C) Quantification of the distribution of projected area for control (open bars) and azithromycin-treated GUVs (filled bars) with class intervals of 70 μm^2. The size distributions were calculated on 530 control and 606 azithromycin-treated GUVs. Bars are centered on the middle of each interval. Azithromycin significantly altered the GUVs distribution, as shown by the Kolmogorov–Smirnov test ($p<0.0001$).

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

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

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

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

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

Calculations were performed on an Intel® Pentium® 4, CPU 3.80 GHz, 4.00 Go of RAM, using Z-TAMMO software. Graphs were drawn using WinMGM (Ab Initio technology, Obernai, France).

3. Results

3.1. Azithromycin alters the morphology of DOPC GUVs

As evidenced by direct microscopic examination, GUVs prepared by electroformation showed an initial spherical shape reaching diameter up to ~50 μm (Fig. 3; 0 s), with <3% of vesicles below 5 μm. Addition of 50 μM azithromycin induced reversible fluctuations followed by irreversible loss of the largest vesicles. First, the membrane showed a rapid phase of budding and fission followed by reverse fusion (Fig. 3; 10–30 s). Incomplete back fusion eventually resulted into the progressive disappearance of the largest vesicles within approximately 30 min (Fig. 4A and B). Statistical analysis of size distribution (Fig. 4C) confirmed that the drug induced the loss of the largest GUVs with the concomitant increase of smallest vesicles (centered on 35 μm^2 projected area), from ~40% in controls to ~60% in azithromycin-treated samples. These morphological observations indicated that azithromycin preferentially destabilized membranes with the lowest curvature.

3.2. Azithromycin decreases the bending and the elastic compressibility moduli of DOPC GUVs

As detailed in Materials and methods, the amplitude of thermal fluctuations of a bilayer is related to its bending elasticity, a mechanical property of the membranes gauged by the bending modulus (k_c); the apparent area compressibility modulus (K_{app}) characterizes the energy necessary for the increase of a bilayer surface area. To determine these parameters, the values of α and σ were derived from Eqs. (2) and (3), respectively. The bending modulus (k_c) was deduced from the slope of the linear relationship between the logarithm of the tension ($\dot{\sigma}$) and the relative excess area difference (α) for low values of the tension. The elastic compressibility modulus (K_{app}) was deduced from the slope of the linear relationship between ln σ and α for values of σ above 0.5 mN/m.

The effects of azithromycin on these biophysical parameters are shown at low (Fig. 5A) and high σ values (Fig. 5B). For control vesicles, the bending modulus k_c was estimated at 23.1±3.5 k_BT; the elastic compressibility modulus, K_{app}, was evaluated at 176±35 mN/m. Upon azithromycin exposure, the bending modulus, k_c, decreased by ~55% to 10.6±4.5 k_BT and the elastic compressibility modulus (K_{app}) decreased by ~35% to 113±25 mN/m, indicating that azithromycin insertion in lipids reduced requirements of both energy for thermal fluctuations and of stress to stretch the bilayer.

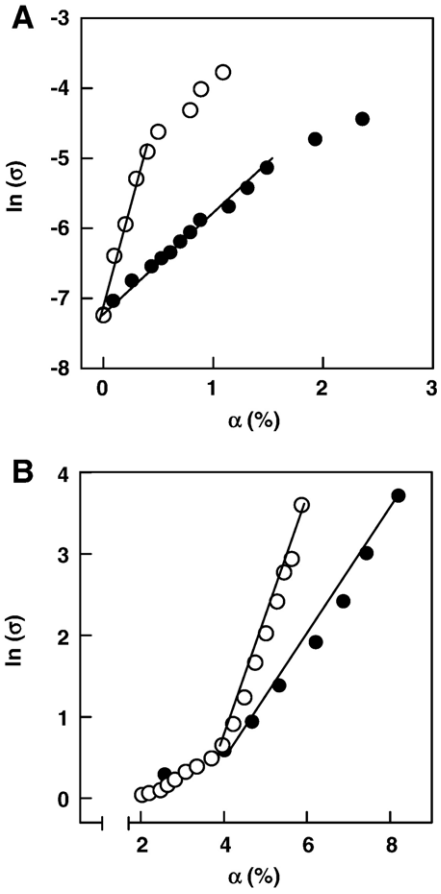

Fig. 5. Tension (ln σ) versus area increase (α) for control (open symbols) or azithromycin-treated GUVs (filled symbols) at σ < 0.5 mN/m (A) or > 0.5 mN/m (B). The bending modulus (k_c) and the apparent area compressibility modulus (K_{app}) were calculated from the slope of the linear components, represented as straight lines based on Eq. (4). Typical results obtained for one representative out of 25 control and 40 azithromycin-treated GUVs.

3.3. Molecular simulation of the interaction between azithromycin and DOPC bilayers

Molecular modeling, explained in details under Materials and methods, predicted that azithromycin interacts with the membrane interface of DOPC and induces a modification in the DOPC organization (compare panels A and B Fig. 6). Structurally, this modification corresponded to an increase in the area of each lipid molecule, from 68.6 Å^2 for pure lipid to 75.9 Å^2 when azithromycin was present. It should be noted that the calculated area for pure DOPC fitted the mean experimental values [17]. Remarkably, modeling reveals the appearance of a noticeable space around the azithromycin molecule. Since calculations predicted the azithromycin:DOPC assembly inserted in an implicit simplified bilayer, this empty space should be filled with the hydrocarbon chains from the lipids belonging to the upper layer.

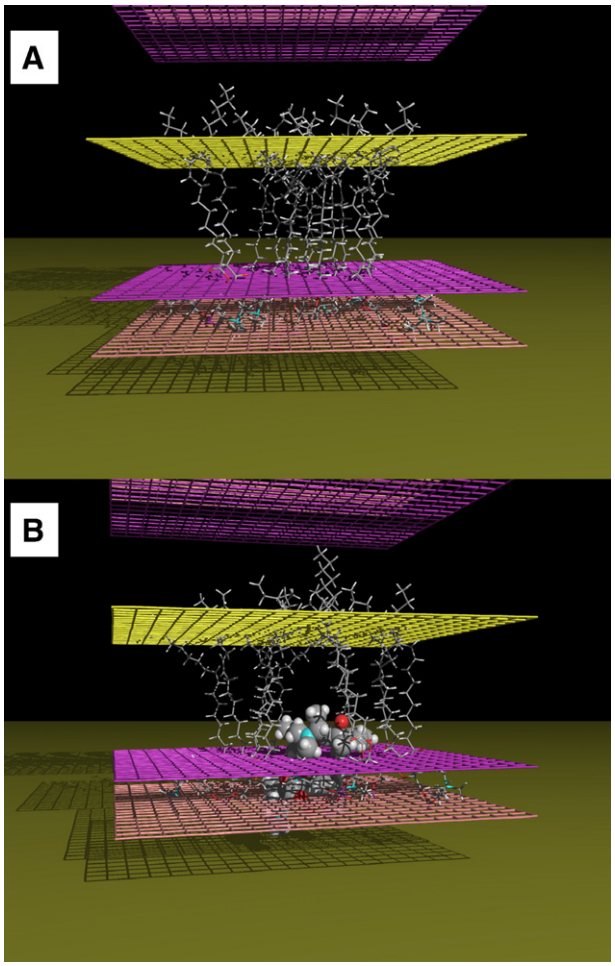

Fig. 6. Assembly of DOPC alone (A) or combined with azithromycin (B) inserted in the membrane as determined by IMPALA method. Yellow plane = bilayer centre ($z=0$); magenta plane = phospholipid acyl chain/polar headgroup interface at $z=13.5$ Å from the centre; pink plane = phospholipid:water interface ($z=18$ Å). Azithromycin is represented in CPK (Corey–Pauling–Kaltum) mode. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Thermodynamically, azithromycin was predicted to decrease the interaction energy between DOPC molecules, from −15.5 Kcal mol^{-1} to −11.5 Kcal mol^{-1}. The energy of interaction between azithromycin and DOPC was predicted at −33.2 Kcal mol^{-1}. Taken together, results of this *in silico* approach indicated that azithromycin insertion in lipids decreases the energy of interaction between DOPC molecules and increases the mean surface occupied by each phospholipid molecule.

4. Discussion

Self assembly of biological structures is an intricate process where a variety of intermolecular forces interplay. The major force holding membrane lipid molecules together are the London–Van der Waals forces. Van der Waals interactions in general are attractive forces which are not simply based on electrostatic interactions. They are attributed to electromagnetic interactions, occurring by fluctuations of charges. These forces, together with the configurational entropy of the hydrogen bond networks, are involved along the acyl chains and are responsible for the organization of phospholipids into various lyotropic structures. In addition to this global organization, lipid bilayers can be characterized by their dynamic equilibrium thermal fluctuations including stretching and bending deformations. Lipid: lipid interactions and mechanical properties of membranes, especially bending rigidity, can be affected by incorporation of amphiphilic molecules, such as azithromycin, which perturbs the lipid packing. Recent atomic force microscopy analysis have revealed that the macrolide antibiotic, azithromycin, lead to an erosion of DPPC gel phase domains in DOPC fluid phase matrix [1,4].

To further examine if these alterations were related to impairment by azithromycin of membrane elastic properties, especially those of the bulk fluid phase of DOPC, we here investigated the effect of this antibiotic on the bending modulus (k_c) and the apparent compressibility modulus (K_{app}) using the powerful micropipette aspiration technique. This approach, originally developed by Evans et coll. [10,11], has been largely used to derive biophysical parameters by the analysis of induced deformation of large artificial vesicles or living cells. Experimental determination of elastic moduli have provided major information about the adhesive properties of cells [18,19] as well as the effect induced by (i) change in membrane lipid composition [20], temperature [20] or mechanical stress [21] or (ii) interaction of exogenous molecules like peptides [22], proteins [23], drugs [24], or alcohols [25]. Our results using this approach to investigate the effect of azithromycin on the membrane elastic properties can be related to the ability of this antibiotic to modify the DOPC vesicles shape, as monitored by conventional microscopy, and to the change of the mean surface area occupied by one DOPC molecule together with the energy of interaction between DOPC molecules, as predicted *in silico* after molecular modeling.

Due to their single membrane and large size, giant unilamellar vesicles (GUVs) represent an advantageous model system mimicking cell membranes for both physical and biological investigations [10,26,27]. In particular, the low membrane curvature of GUVs better mimics that of flat domains of the plasma membranes than conventional small vesicles exhibiting a higher curvature. In addition, at low curvature, stochastic fluctuations should enhance penetration of exogenous molecules [28]. Furthermore, GUVs can be visualized by conventional microscopical methods and are very sensitive to environmental parameters such as temperature variations, electric field, mechanical constraints, local modification of their composition or insertion of exogenous compounds [29,30], all of which may affect the energetic equilibrium of the bilayer. Moreover, studies on individual GUVs provide informations on their structure and physical properties as an effect of time and spatial coordinates, that cannot be obtained by the population-based studies of lipid vesicles in suspension [31].

Conventional microscopy readily showed that addition of azithromycin immediately triggered visible GUVs fluctuations: the shape of the vesicles was modified, so that a new energy minimum would be reached and the vesicle started budding. However, this change of shape appeared to be not sufficient for the bilayer to keep a constant minimum energy, and DOPC

molecules probably underwent rearrangement. The mechanism that controls the budding remains uncertain. Flip-flop, which is extremely slow for phospholipids (half-time of several hours [32]), is probably not involved in this process. After 30 s, the bilayer recovered a spherical shape corresponding to the initial shape (i.e. before the addition of the antibiotic). After a longer time interval (30 min), the largest GUVs had disappeared. It is likely that the drug, added outside GUVs, is unable to cross the membrane bilayer, thus preferentially inserts into the outer membrane layer and affects the bilayer couple [33]. In this view, the shape of an individual GUV is determined by the minimum bending energy for a given area, A, a given volume, V, and a given area difference between the two monolayers in the bilayer membrane area, ΔA. In the presence of azithromycin, the increase in the area difference between the two leaflets of the bilayer, ΔA, without change of V, would lead to a rearrangement of the lipid molecules until destruction of the vesicle. Since fluctuations of the largest vesicles should lead to considerable movement of the membrane, as indeed shown by microscopy, a higher proportion of foreign molecules should be entrapped in the external monolayer and affect the equilibrium of the entire bilayer, explaining the preferential disappearance of the largest vesicles.

The effect of azithromycin on the vesicle shape prompted us to measure the elastic bending constants of lipid bilayers, and especially, to compare the bending modulus and the apparent area compressibility modulus in the absence and presence of azithromycin. The elastic properties of membranes modulate lipid:lipid interactions [34], and also affect the lateral compressibility [11,35] in a way that might have functional consequences, e.g. for membrane insertion of proteins [23] or drugs [24,35]. Only very few studies have addressed this possibility. The bending modulus (k_c) of untreated DOPC GUVs was measured as 23.1 ± 3.5 k_BT, in excellent agreement with the literature [20,36] when taking into consideration that differences in the mode of preparation of GUVs, in the temperature during the formation of GUVs, and in the composition of the buffer can all induce strong discrepancies in the measured elastic parameters [20,36]. The bending modulus, k_c, decreased two-fold in presence of azithromycin, indicating that insertion of the antibiotic in the DOPC bilayer reduced the energy requirements for thermal fluctuations. This decrease could be due to a modification of the spontaneous curvature and/or to a higher lateral diffusion of the constituents of the bilayer [36]. This effect was indeed observed together with a higher compressibility of membrane, reflecting that a lower stress is required to stretch the bilayer (K_{app} decreased from 176 ± 35 mN/m in controls, again in good agreement with the literature [36], down to 113 ± 25 mN/m in presence of azithromycin). Since the apparent area compressibility modulus (K_{app}), is itself related to the packing of the constituents of the bilayer, its decrease upon azithromycin must reflect a reduction in the lipid bilayer density and the smoothing of thermal fluctuations. These changes could affect the membrane thickness and it should be interesting to further investigate the effect of azithromycin on this parameter using approaches like Reflection Interference Contrast Microscopy (RICM) [37] or X-ray diffraction [38].

These conclusions are confirmed independently by *in silico* modeling using, the IMPALA restraint field, developed by one of the authors [16]. This method allows one to study the interactions between a drug and a lipid bilayer using simple restraint functions designed to mimic the major properties of the membrane, including the solvent hydrophobicity and the lipid perturbation. This approach has been already useful to predict the insertion of tilted peptide [39] and to design *de novo* a fusogenic peptide made of non-natural aminoacids [40]. Here, we show by molecular modeling that azithromycin inserts at the interface of lipids. Remarkably, a noticeable space was observed around the azithromycin molecule in the lipid monolayer. Since the calculations on the interaction of azithromycin with DOPC are made on a monolayer, the empty space around azithromycin should be filled with the hydrocarbon chains from the lipids belonging to the upper layer. This cannot be, however, predicted using the currently modeling approach available. Indeed, calculations are made in two steps. The first one is to surround azithromycin with lipid molecules, forming a monolayer. This monolayer (one layer of lipids+azithromycin) is then positioned in an implicit bilayer (i.e. only the properties of the bilayer are simulated by an empirical function, see Methods). Thus, the physical effect of azithromycin molecule on the lipid molecules is only predicted in the monolayer and the effects on the entire bilayer cannot be evaluated. Combined with experimental data, our study, however, clearly demonstrates that azithromycin insertion within the lipid bilayer reduces its cohesion and increases the mean surface occupied by each lipid molecule.

Micropipette experiments revealed a marked decrease of the elastic moduli of DOPC lipid bilayers upon exposure to azithromycin, with both k_c and K_{app} being strongly affected. Morphological studies on DOPC GUVs showed that azithromycin leads to the destruction of the largest ones, probably due to lipid vesicles reorganization. These effects may be due to an increase of the available space between hydrophobic chains, enhancing their mobility, in agreement with the partition of azithromycin within the membrane interface, and to the decrease of the interaction energy between DOPC molecules in the presence of azithromycin, as predicted by molecular modeling. The excellent agreement between theoretical and experimental approaches also underlines the potency of molecular modeling to predict the effect of drug insertion on lipid membrane properties.

The decrease of the cohesion of DOPC bilayer induced by azithromycin antibiotic, as observed in this study, together with the fluidification of DPPC [4] and the probable preferential accessibility of azithromycin at the interface between DPPC and DOPC [41–43], might explain why azithromycin is able to erode the lipid domains of DPPC and to facilitate the merging of DPPC gel phase into a DOPC fluid matrix, as observed by atomic force microscopy [1]. Our findings thus provide new molecular insights of the interaction between a macrolide antibiotic, as drug model, and an artificial lipid membrane, made of pure DOPC. Since cholesterol markedly affects the membrane curvature and bending elasticity [44,45], these studies should be extended to GUVs with more complex lipid composition, for a better understanding of relevant effects of azithromycin and other drugs for cellular membranes.

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