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Interaction of the macrolide azithromycin with phospholipids. II. Biophysical and computer-aided conformational studies

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Abstract

In a companion paper, we show that azithromycin causes a lysosomal phospholipidosis in cultured cells, binds in vitro to negatively charged bilayers without causing aggregation or fusion, and inhibits lysosomal phospholipase A_1. In this paper, we show that azithromycin decreases the mobility of the phospholipids in negatively charged liposomes (using ^{31}P nuclear magnetic resonance) and that it increases the fluidity of the acyl chains close to the hydrophilic/hydrophobic interface, but not deeper into the hydrophobic domain (assessed by measuring the fluorescence polarization of trimethylammonium-diphenylhexatriene and diphenyhexatriene, respectively). Computer-aided conformational analysis of mixed monolayers of azithromycin and phosphatidylinositol shows that the drug can be positioned largely in the hydrophobic domain, but close to the interface, with the macrocycle facing the C_1 of the fatty acids (allowing the N_{9a} endocyclic tertiary amine to interact with the phospho-groups), the cladinose located on the hydrophobic side of the lipid/water interface and the desosamine projected into the hydrophobic domain. This position is consistent with the experimental data. Analysis of virtual molecules shows that this unanticipated behavior is due to the shielding of the ionizable $N_{3'}$ amino-group in the desosamine by methyl-groups, and to the wide dispersion of hydrophobic domains all over the molecule. The interaction of azithromycin with phospholipids may account for some of its unusual pharmacokinetic properties and for its potential to cause lysosomal phospholipidosis.

Keywords: Azithromycin; Phospholipid; Fluorescence polarization; NMR spectroscopy, ^{31}P; Conformational analysis; Gentamicin

1. Introduction

Azithromycin is a dicationic macrolide antibiotic with exceptionally high levels of tissue accumulation, due to its storage in lysosomes. In cultured cells as well as in animals treated with large doses, azithromycin causes the development of a lysosomal phospholipidosis (Shepard et al., 1992; Van Bambeke et al., 1996a; Montenez, 1996). Using liposomes in vitro, we showed in the companion paper that azithromycin binds to negatively charged bilayers at acidic pH and causes a dose-dependent inhibition of lysosomal phospholipase A_1. This effect is probably due to neutralization of surface negative charges which are critical for enzyme activity (for a discussion of the charge dependency of phospholipase A_1 activity, see Mingeot-Leclercq et al., 1988, 1995a; Piret, 1993). We also showed, however, that membrane-bound azithromycin fails to cause aggregation or fusion of liposomes, in sharp contrast to what is observed with other dications, such as Ca^{2+}, Mg^{2+} or bis(β-diethylaminoethylether)hexestrol (DEH) (Wilschut et al., 1981; Mingeot-Leclercq et al., 1989). This suggests that the membrane-bound antibiotic is unable to bridge adjacent membranes and is, therefore, deeply inserted in the bilayer, in spite of its dicationic character.

In the present paper, we examine in more details the molecular interactions between azithromycin and phospholipids. We used two complementary approaches in parallel, viz., an experimental evaluation of the capacity of azithromycin to interact with the polar and apolar domains of the membrane, respectively, together with a conforma-

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tional analysis of azithromycin at a the lipid/water interface and in interaction with acidic phospholipids. The latter approach uses computational procedures which have been successfully applied to a series of drugs, peptides and proteins showing distinct types of interactions with membrane bilayers (Tulkens et al., 1990; Chatelain and Brasseur, 1990; Brasseur et al., 1988a,b, 1992).

2. Materials and methods

2.1. Experimental studies

2.1.1. Drug solubilization

Because azithromycin is sparingly soluble in water at neutral pH, a solution was prepared by dissolving ≈ 50 mg of the free base in 1 ml 0.1 M HCl. The pH of the solution was thereafter adjusted at 5.4 by the addition of NaOH. Gentamicin sulfate (a mixture of C_1, C_{1a} and $C_{2/2a}$ components) was directly dissolved in 40 mM acetate buffer, pH 5.4 at a concentration of 50 mg/ml. These stock solutions were thereafter diluted to the concentration necessary for each experiment by the addition of an appropriate volume of 40 mM acetate buffer, pH 5.4.

2.1.2. Liposome preparation

Large unilamellar vesicles (LUV) were prepared exactly as described by Van Bambeke et al. (1993) and presented an average size of ≈ 100 nm as measured by light scattering spectroscopy (Van Bambeke et al., 1995, 1996a). Multi-lamellar vesicles (MLV) were obtained by the same procedure but omitting the extrusion steps. They presented a mean diameter of > 2000 nm as determined by light scattering spectroscopy. Analysis of these samples using the size distribution processor revealed, however, the presence of 2 populations of vesicles, viz., 15% presenting a mean diameter of 350 ± 80 nm and 85% presenting a mean diameter of 3800 ± 820 nm. All liposomes had the same composition [cholesterol, phosphatidylcholine, sphingomyelin and phosphatidylinositol (5.5:4:4:3 molar ratio)], and were prepared in 40 mM acetate buffer, pH 5.4. The actual phospholipid content of each preparation was determined by phosphorus assay (Bartlett, 1959) and the concentration of liposomes was adjusted accordingly for each type of experiment.

2.1.3. ^{31}P nuclear magnetic resonance

The residual chemical shift anisotropy ($\Delta\sigma$) of the phosphorus atoms of phospholipids is the chemical shift difference between the high field peak and the low field shoulder in the spectra of bilayer phospholipids (Seelig, 1978). It was determined in the present study on large multi-lamellar vesicles. Since the spectra of our mixed liposomes did not always show a well defined shoulder at low field, we determined the $\Delta\sigma$ values using the difference between the high field maximum chemical shift and the isotropic shift, which corresponds to one-third of the $\Delta\sigma$ value (Seelig, 1978). 1 ml of sample, containing 20 mg of lipids (31.4 mM total lipids), was mixed with 200 μl of D_2O (for locking on the deuterium signal, resulting in stabilization of the field frequency ratio), before the addition of the drug. Spectra were recorded upon warming of the sample from 33 to 70°C with a Bruker WM 250 Fourier Transform spectrometer (Bruker, Wissembourg, France), operating at 101.3 MHz. Typical Fourier transform parameters were: 2500 scans; 45° (12 μs) flip angle; 25 kHz spectral width; 4K data points; 0.8 s interpulse time. Proton decoupled spectra were obtained by using powergated decoupling to minimize dielectric heating. High-power decoupling (5 W) was applied during acquisition and low-power decoupling (0.5 W) during the delay. A line broadening of 50 Hz was applied to the free induction decay before Fourier transformation.

2.1.4. Fluorescence polarization studies

Fluorescence polarization studies were performed on large unilamellar vesicles at a final concentration of 0.2 mg/ml (0.314 mM total lipids). Incorporation of fluorescent markers (at a molar ratio to the lipids of 1 : 250) was obtained by a vigorous mixing followed by a preincubation at 37°C during 1 h. Two markers with distinct localization in the membrane were used in a comparative fashion [diphenylhexatriene (DPH), a totally hydrophobic probe, which penetrates in the deepness of the membrane; and trimethylammoniumdiphenylhexatriene (TMA-DPH), which spans the hydrophilic/hydrophobic interface because of its amphiphilic character; see Kitagawa et al. (1991)].

Labeled large unilamellar vesicles were mixed with the drug, incubated at 37°C for 30 min, brought to 60°C in 15 min, and maintained at that temperature during 5 min for stabilization before starting the measurements. The fluorescence emitted in the planes parallel (I_{par}) and perpendicular (I_{per}) to that of the polarized excitation light was then measured while the samples were cooled down to 10°C at a rate of 50 C°/h. Results are expressed as $P = (I_{par} - I_{per})/(I_{par} + I_{per})$. Fluorescence was measured on a LS-50 Perkin-Elmer fluorimeter (Perkin-Elmer, Beaconsfield, UK), equipped with a special adaptor for polarization measurements, and operating at an excitation wavelength of 365 ± 5 nm (for DPH) or 360 ± 5 nm (for TMA-DPH) and an emission wavelength of 427 ± 3 nm (for DPH) or 435 ± 4 nm (for TMA-DPH). The sample was kept under gentle stirring throughout the experiment and its temperature was continuously monitored by a sensor placed into the measuring unit coupled with a programmable circulator bath DC5 (Haake, Karlsruhe, Germany).

2.2. Theoretical studies

The method used implies three successive steps which are: (1) the calculation of the conformation of the isolated

drug molecule based on X-ray data; (2) the calculation and orientation of the drug molecule and of the phosphatidylinositol molecule at a lipid/water interface; and (3) finally, the calculation and orientation of the drug in a lipid monolayer (assembly procedure).

2.2.1. Conformation of the isolated molecule

Our earlier studies with aminoglycosides and other membrane-interacting polycations [see Van Bambeke et al. (1993) and the references cited in that paper] used initial drug conformations determined by calculation only. Because of the complexity of the structure of azithromycin, it was thought safer to use the structural coordinates determined by X-ray crystallography. In our experience (Brasseur et al., 1982), these coordinates provide a satisfactory starting point while increasing the probability to obtain a stable structure after optimization procedure. The coordinates of azithromycin were communicated to us by Dr. M. Vinkovic (Pliva research Institute, Zagreb, Croatia); originally, we used the coordinates stored in the Cambridge Structural Database as the refcodes GEGJAD (Cambridge Structural database, 1994, version 5.07, Cambridge Crystallographic Data Center, 12 Union Road, Cambridge, UK); however, when the corresponding three-dimentional structure was examined by reference to literature data (Kirst and Sides, 1989), it appeared that it corresponded to the enantiomer of azithromycin; Dr. M. Vinkovic informed us that a technical mistake in the laboratory where the original X-ray data on azithromycin crystals had been obtained had resulted in an inversion of coordinates; the correct coordinates actually used in this paper are, thus, the inverted values of those stored in the Cambridge Structural Database (see Sheldrick et al., 1995). The data were put in a Hyperchem 4.0 compatible format for further manipulation. Full protonation of the azithromycin molecule was modeled by adding hydrogen atoms on each of amino functions and by giving 2 positive charges to the whole molecule. The repartition of these total charges on individual atoms was performed using the CNDO procedure of Hyperchem 4.0. The molecules were then minimized by optimisation of their configuration by the MM + procedure (Fletcher-Reeves algorithm) of Hyperchem 4.0. The configuration was considered as of lowest energy when the gain between two calculations was ≤ 0.2 kcal/(Å.mol). The same procedure was applied for the virtual molecules used in the analysis (see below).

For phosphatidylinositol, we used the coordinates described previously by Brasseur et al. (1984) and considered the molecule to consistently carry one negative charge on its phospho-group, assuming a pK_a of ≈ 2.5 for the free-acid function of phosphatidylinositol (Traüble and Eibl, 1974).

All other values used for the valence angles, bond lengths and atomic charges are those currently used in conformational analysis studies (Hopfinger, 1973) and are similar to the values used in computer modeling (Weiner et al., 1984; McCammon and Harvey, 1987; De Loof et al., 1991). The most probable configurations were taken as those yielding the lowest internal energy.

2.2.2. Conformation and orientation of the isolated molecule at the lipid / water interface

This step used a strategy reviewed by Brasseur (1990). To simulate the membrane interface, the dielectric constant was assumed to increase linearly from 3 to 30 along a z-axis perpendicular to the putative interface. The total conformational energy of the molecule at the lipid/water interface was empirically calculated as the sum of all contributions resulting from local interactions, i.e. Van der Waals energy, electrostatic interaction, transfer energy and torsional potential. The transfer of energy for atoms has been determined and published in Brasseur (1991). The molecule was finally oriented with the segment joining the hydrophilic and hydrophobic centers perpendicular to the interface (Brasseur et al., 1986). Distance between the hydrophilic and hydrophobic centers (Δ), and the hydrophobic/hydrophilic balance (ϕ) were thereafter determined as described in Brasseur et al. (1986) and Brasseur (1990).

2.2.3. Interaction of the drug with lipids

We used the Hypermatrix Method to surround one molecule of azithromycin with an increasing number of phosphatidylinositol molecules. This method is a modification of the sequential method used previously to surround one molecule of drug or of peptide with successive lipid molecules (Brasseur et al., 1981). It is based on a non-relaxed strategy in which the molecular structures of all compounds are fixed through the hypermatrix procedure (for review, see Brasseur, 1990). The methodology allows to explore the energy levels at fairly large distance of each point of low energy, so that the probability of determining the truly lowest point of energy is very large.

The conformational analysis was performed using a Olivetti computer equipped with Pentium Intel microprocessors, using PC-TAMMO + (Theoretical Analysis of Molecular Membrane Organization) and PC-MSA + (Molecular Structure Analysis) softwares (Brasseur, 1990). Graphs were drawn with the Win-MGM (Molecular Graphics Manipulation) program. These programs and information on their characteristics are available from their author (R.B.).

2.3. Materials

Azithromycin was supplied as dihydrate free base by Pfizer (Groton, CT, USA). Gentamicin (mixture of components C_1, C_{1a} and $C_{2/2a}$ in a molar ratio of $\approx 27:21:52$) was supplied as sulfate salt for research purpose by Schering Plough (Kenilworth, NJ, USA). Glycerophospholipids (egg yolk phosphatidylcholine, wheat germ phosphatidylinositol) were purchased from Lipid Products (Nr Redhill,

UK), as grade-1 products, and bovine brain sphingomyelin and cholesterol from the Sigma (St. Louis, MO, USA). Diphenylhexatriene (DPH) and trimethyldiphenylhexatriene (TMA-DPH) were obtained from Molecular Probes (Eugene, OR, USA). Other reagents were obtained from E. Merck (Darmstadt, Germany) and were of analytical grade.

3. Results

3.1. ^{31}P-NMR spectroscopy (large multi-lamellar vesicles)

To evaluate the capacity of azithromycin to modify the mobility of the phospholipids, we examined the modification of ^{31}P-NMR spectra of large multi-lamellar vesicles upon an increase in temperature.

Typical spectra of control large multi-lamellar vesicles (31.4 mM total lipids) and large multi-lamellar vesicles incubated with azithromycin (5.6 mM) or gentamicin (1.1 mM) are shown in Fig. 1. At 33°C, control liposomes showed a broad spectrum characterized by a maximum at high field and a shoulder at low field; a weak shoulder at high field is also observed resulting from differences in chemical shift anisotropy. A temperature increase caused a progressive reduction of $\Delta\sigma$ in addition to a definite upsurge of a narrow peak at 0 ppm, the proportion of which remained, however, rather low. The addition of azithromycin caused almost no change in the shape of the spectra at 33°C but caused a lesser reduction of the chemical shift anisotropy upon warming. Moreover, no or only minimal upsurge of the isotropic signal developed upon warming. The addition of gentamicin caused a very similar effect, both with respect to the decreased reduction of the chemical shift anisotropy upon warming and the drastic reduction of the isotropic signal at high temperature.

Fig. 2 shows the calculated values of the effective chemical shift anisotropy ($\Delta\sigma$) in the different conditions tested. A sharp decrease was seen when control liposomes were warmed from 33°C to 70°C. This decrease was significantly lower ($P < 0.05$) in the presence of either azithromycin or gentamicin with an essentially similar effect of the two drugs.

3.2. Fluorescence polarization studies (large unilamellar vesicles)

To investigate the influence of azithromycin on the zones of the hydrophobic domain immediately adjacent to the hydrophilic/hydrophobic interface and those deeper inside the inner part of the membrane, we examined its effect on the degree of fluorescence polarization of two probes which insert themselves in bilayers at different levels, viz. trimethylammoniumdiphenylhexatriene (TMA-DPH), and diphenylhexatriene (DPH). The first one localizes beween the alkyl chains but spans the hydrophilic/hydrophobic interface whereas the second one is totally hydrophobic. Fig. 3 shows that the degree of polarization of the two probes decreased in a linear fashion in control large unilamellar vesicles when the temperature of the sample was increased (correlation coefficient > 0.99 for

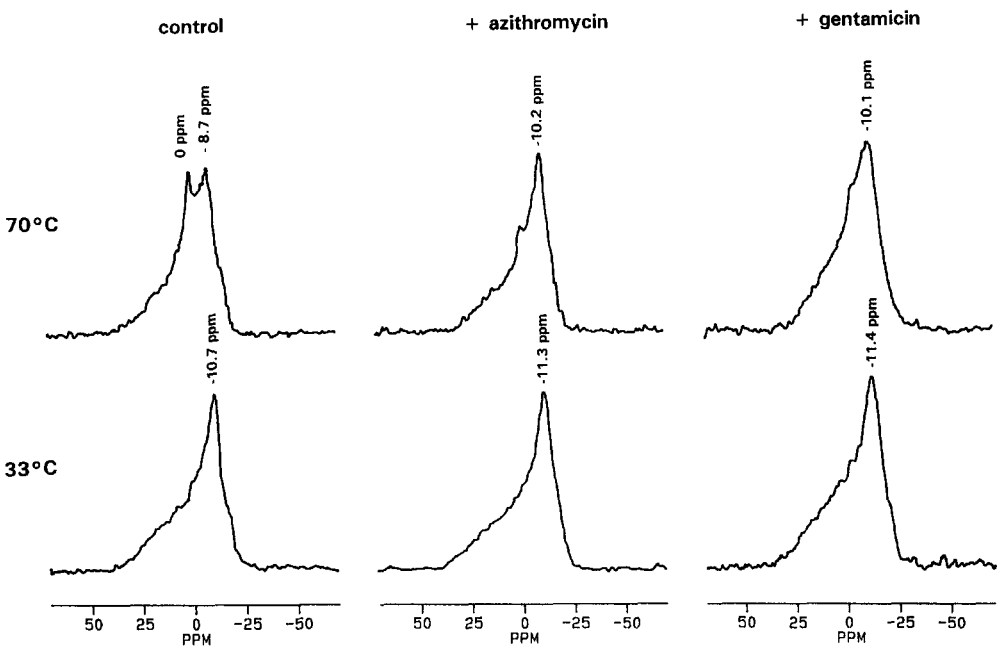


Fig. 1. ^{31}P-NMR spectra of large multi-lamellar vesicles obtained at 33 and 70°C, in the absence (left) or in the presence of azithromycin (middle) and gentamicin (right) at a concentration of 5.6 and 1.1 mM (corresponding to drug:phospholipid molar ratios of 0.270 and 0.053, respectively). The liposomes (31.4 mM in lipids) were made of a mixture of cholesterol, phosphatidylcholine, sphingomyelin and phosphatidylinositol (5.5:4:4:3 molar ratio) and prepared in 40 mM acetate buffer pH 5.4. The isotropic signal is set at 0 ppm as a reference and the figure shows the chemical shift values of the peak at high field.

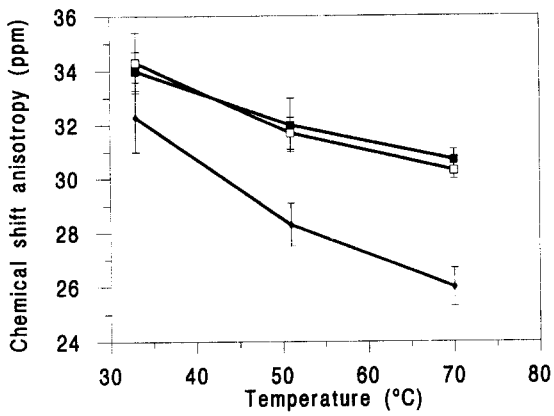


Fig. 2. Variation of the effective chemical shift anisotropy ($\Delta\sigma$) in ^{31}P-NMR of large multi-lamellar vesicles as a function of temperature. The liposomes were prepared and treated as in Fig. 1. The different curves correspond to control liposomes (♦) or liposomes incubated 1 h at 37°C with azithromycin (■) or gentamicin (□). Each sample was successively examined at increasing temperatures and recordings were made over ≈ 1 h at each temperature investigated. $\Delta\sigma$ values (±S.D.; $n = 3$–7 independent determinations at variable temperatures) were calculated as indicated in Section 2.

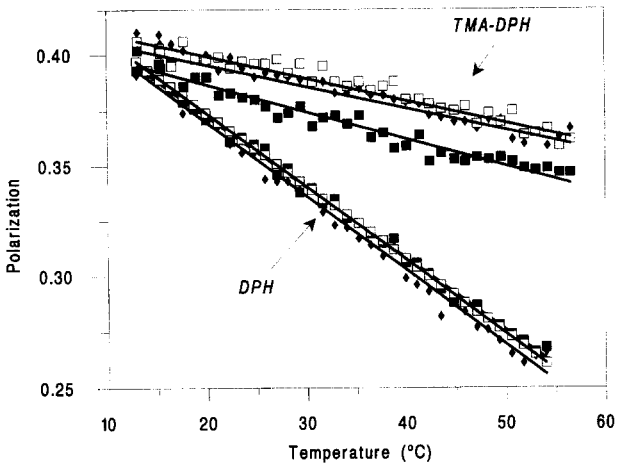
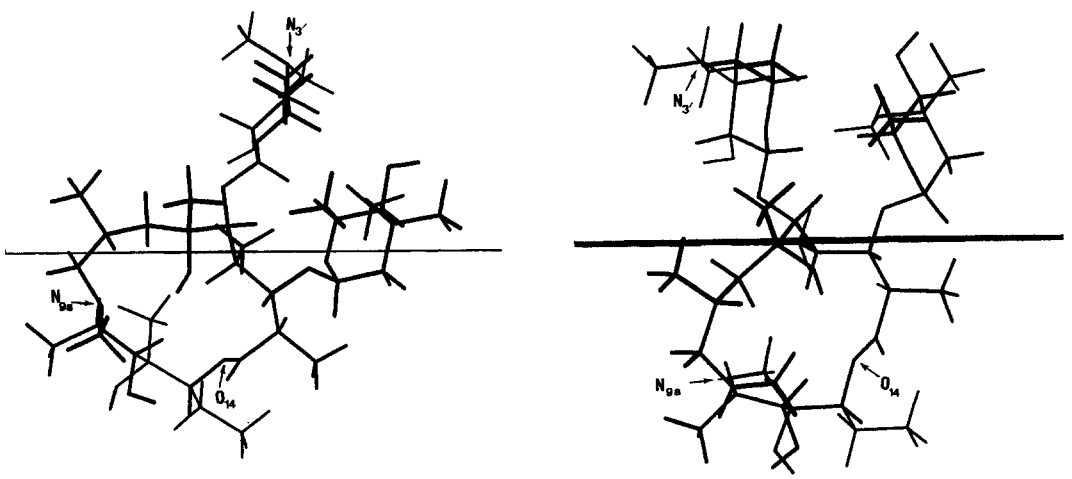


Fig. 3. Variation of the polarization of the fluorescence of TMA-DPH or DPH incorporated in large unilamellar vesicles (molar ratio to the lipids 1:250) made of cholesterol, phosphatidylcholine, sphingomyelin and phosphatidylinositol (5.5:4:4:3 molar ratio). (♦), control vesicles; (■) vesicles incubated with azithromycin; (□), vesicles incubated with gentamicin (molar ratio to total phospholipids 0.03 for both drugs). Each point is the mean value of 4 independent experiments but the S.D. are not shown for sake of clarity. The straight lines represent the corresponding linear regression (correlation coefficient > 0.99 for experiments with DPH and > 0.94 for experiments with TMA-DPH).

experiments with DPH and > 0.94 for experiments with TMA-DPH). The variation of polarization values with TMA-DPH was, however, lower than with DPH, which we interpret as indicating a lesser increase in mobility of the alkyl chains close to the interface upon warming in comparison to what occurs in the deeper hydrophobic domain.

The addition of azithromycin significantly reduced the degree of polarization of TMA-DPH of ≈ 0.01 at all temperatures, which would mean an increase in fluidity equivalent to what would be obtained by an increase in the



Azithromycin	E_{tr}^{Pho} (kcal/mol)	E_{tr}^{Phi} (kcal/mol)	ϕ	Δ (Å)
unprotonated	- 150.2	45.2	0.521	0.46
protonated	- 150.2	47.3	0.502	0.39

Fig. 4. Skeleton views of the most probable conformers of azithromycin under its non-protonated (left) and protonated (right) forms at a water/lipid interface (indicated by the horizontal bar; the lipid phase being above). The position of the two amino functions in the drug and the heterocyclic oxygen are indicated by arrows, and the numbers correspond to their position as indicated in the structural formula shown in Fig. 5. The figure also shows the key conformational parameters used in the calculation of the conformers (E_{tr}^{Pho}, hydrophobic transfer energy; E_{tr}^{Phi}, hydrophilic transfer energy; ϕ, hydrophobic, hydrophilic balance; Δ, distance between hydrophobic and hydrophilic centers).

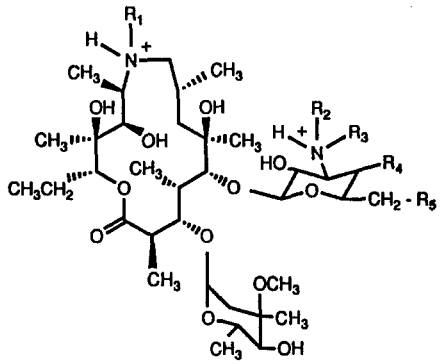
temperature of 9°C. In contrast, azithromycin did not alter significantly the degree of polarization of DPH over the whole range of temperatures investigated. Gentamicin, added in a drug/lipid molar ratio similar to that of azithromycin did not alter the degree of polarization of either probes.

3.3. Conformational analysis

3.3.1. Isolated molecules at lipid / water interface

The most probable conformers of the protonated and the unprotonated forms of azithromycin are shown in Fig. 4, together with their pertinent conformational parameters in relation to their position at the interface. Interestingly enough, these values were all very close, revealing first that azithromycin is largely a hydrophobic molecule, whether protonated or not. Moreover the hydrophobic and hydrophilic centers are at a very short distance very close to one another. This indicates that the hydrophobic and hydrophilic subdomains are always very close and/or intermingled (in contrast, DEH, a dicationic amphiphile which tends to adopt an hair-pin conformation where the two ionizable groups can be placed on one side of the molecule whereas the other side is entirely hydrophobic, shows a distance (Δ) of 2.38 Å between hydrophobic and hydrophilic centers (see Mingeot-Leclercq et al., 1989). When the molecule is under a neutral form, its macrocycle was largely in the hydrophilic zone but close to the interface. The C_5-C_8 part of this cycle, however, stays in the hydrophobic zone, but the hydroxyl in position 6 is nevertheless oriented towards the aqueous phase. The cladinose is buoyant at the interface whereas the unprotonated desosamine is entirely located in the hydrophobic zone. When the drug is ionized (in N_{9a} and $N_{3'}$), only minor changes of conformation, position and orientation were seen. The $N_{3'}$ of the desosamine still remained entirely in the hydrophobic zone, but the macrocycle (with its N_{9a} aminated group) was now found almost entirely in the hydrophilic zone. Due to the rotation of the molecule, however, the cladinose was pushed deeply in the hydrophobic zone, including its 4″ hydroxyl-group (hydroxyl in position 4 in the cladinose), so that the overall equilibrium position of the molecule at the interface was barely modified.

Because it seemed at first sight unreasonable to assume that the protonated form of azithromycin could be so hydrophobic, we analyzed in more details the role of the ionization of the amino-groups, by constructing virtual molecules to obtain orderly changes in hydrophilicity and in the distance between the hydrophilic/hydrophobic cen-



Derivative	R_1	R_2	R_3	R_4	R_5	E_{tr}^{Pho} (kcal/mol)	E_{tr}^{Phl} (kcal/mol)	ϕ	Δ (Å)
Azithromycin	CH_3	CH_3	CH_3	H	H	- 150.2	47.3	0.502	0.39
DA-1	CH_3	H	H	H	H	- 138.9	49.3	0.449	0.53
DA-2	H	H	H	H	H	- 133.2	50.4	0.422	0.47
DA-3	CH_3	H	H	NH_3^+	H	- 138.3	55.5	0.397	1.11
DA-4	CH_3	H	H	NH_3^+	NH_3^+	- 136.2	61.6	0.345	1.59

Fig. 5. Structural formula of azithromycin and of the virtual derivatives used for the conformational analysis. The numbering of the atoms follows the commonly accepted convention for erythromycin and erythromycin derivatives [numbering is made counter-clockwise from the carbonyl adjacent to the lactonic oxygen and giving a 9a number to the additional N in the cycle (Bright et al., 1988; Kirst and Sides, 1989)]. Other authors have given number 10 to this N (Djokic et al., 1987, 1988). The systematic name of azithromycin, following strictly the IUPAC rules (numbering the atoms in the macrocycle clockwise from the lactonic oxygen and indicating the absolute configuration of each chiral atom in the cycle) is (2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-13-([2,6-didesoxy-3-C-methyl-α-L-ribohexopyranosyl]oxy)-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-([3,4,6,tridesoxy-3-[dimethylamino]-β-D-xylo-hexopyranosyl]oxy)-1-oxa-6-azacyclopentadecan-15-one, where the endocyclic N is in position 6. The figures also show the key conformational parameters used for the study (see legend to Fig. 4 for symbols). All data are for the protonated forms of the compounds examined.

ters. We present here 4 of those virtual molecules, in which we introduced first a demethylation of the existing tertiary amines of azithromycin, and then, added new primary amines in the desosamine (molecules DA-1 to DA-4, see structures and pertinent conformational parameters in Fig. 5). Fig. 6 shows the conformation and position of these virtual molecules with respect to the hydrophobic/hydrophilic interface. Didemethylation of the 3′ amino-group of the desosamine (DA-1), induces only a moderate decrease in the global hydrophobic/hydrophilic balance (due mainly to a decrease in the hydrophobic transfer energy) in comparison with protonated azithromycin. Yet, this modification was sufficient to obtain a marked displacement of the hydrophilic and hydrophobic centers, so that the desosamine was now located totally in the hydrophilic zone. Because of this displacement, the macrocycle, including its tertiary amine, became entirely located in the hydrophobic zone. The virtual molecule DA-2 further examines the influence of the demethylation of the amino-groups of azithromycin by removing the methyl-group in position N_{9a} in the macrocycle. This additional demethylation induces a further de-

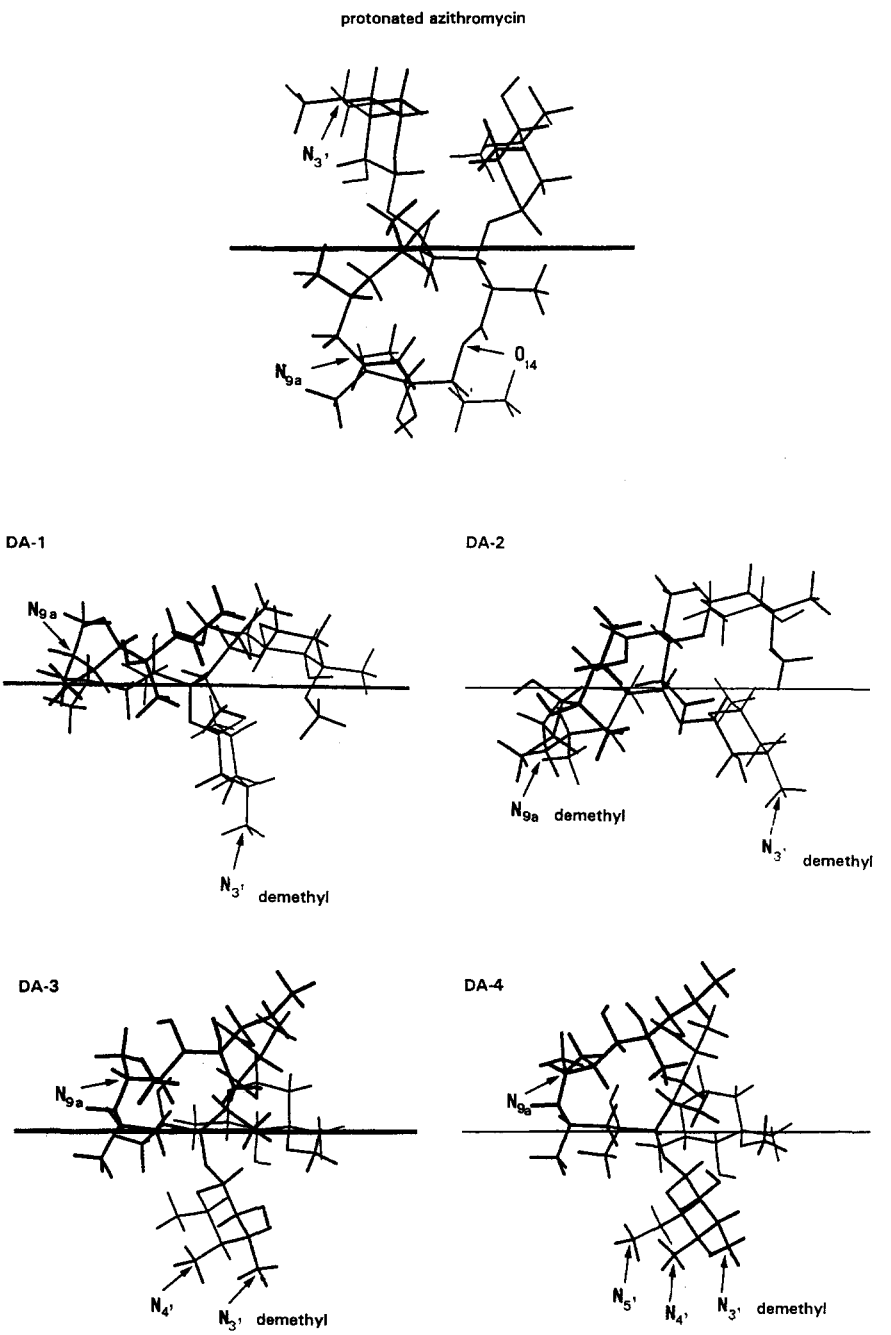


Fig. 6. Skeleton views of the most probable conformers of the virtual derivatives described in Fig. 5, in comparison with azithromycin. All compounds are under their protonated forms. The pattern of each conformer representation and the numbering along the structure are as in Fig. 4.

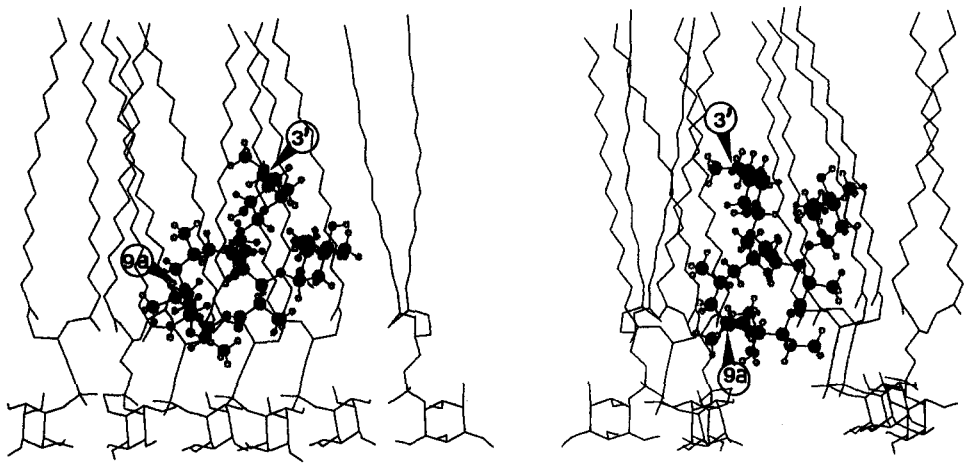


Fig. 7. Views of the mode of assembly of the unprotonated (left) and protonated (right) forms of azithromycin (ball representation) and phosphatidylinositol (skeleton representation). The arrowheads point to the two amino functions of azithromycin (see structural formula in Fig. 5).

crease in the hydrophobic character due to an increase in hydrophilic transfer energy, together with a decrease in hydrophobic transfer energy. It also causes a change in the hydrophilic and hydrophobic centers, resulting in a new position and orientation of the molecule. A large part of the macrocycle, including the new secondary N_{9a} amine, and the $N_{3'}$ amino-group of the desosamine are now both located in the hydrophilic zone, leaving only the cladinose and a minor part of the macrocycle in the hydrophobic zone. It, therefore, appears that the unsuspected position of the protonated azithromycin shown in the first part of this study is likely to result from the masking of its ionizable functions by the methyl-groups.

Because, in all instances, the hydrophobic transfer energy of the molecules was considerably larger than their hydrophilic energy, we placed additional protonated amino-groups in the desosamine, while leaving the $N_{3'}$ group demethylated (DA-3, DA-4; these virtual derivatives must, therefore, be compared to DA-1). Although this addition resulted in a highly polycationic structure, it was insufficient to bring the macrocyclic ring, and its methylated N_{9a} function in the hydrophilic zone, demonstrating the importance of the hydrophobic interactions in this part of the molecule. A critical feature of DA-3 and DA-4, compared with DA-1, and a fortiori azithromycin, is the large distance between their hydrophilic and hydrophobic centers. This is consistent with a reorganization of the hydrophilic/hydrophobic subdomains of the molecules carried by the strong polarization of the desosamine.

3.3.2. Molecules in interaction with phosphatidylinositol monolayers

Both the unprotonated and the protonated forms of azithromycin could be surrounded by up to 6 molecules of phosphatidylinositol, with an interaction energy of 30.8 and 32.7 kcal/mol per mol of lipid, respectively. The mixed monolayers are shown in Fig. 7. As anticipated from the studies of the isolated molecules, the lipids could always be assembled around the drug, in a way that allowed the desosamine, whether protonated or not, to be inserted in the hydrophobic domain. The macrocycle was placed almost *vis-à-vis* of the ester linkage between the fatty acids and the glycerol, still relatively far from the negatively charged phospho-groups. The position of the N_{9a} endocyclic tertiary amine, however, was such that it could interact with those phospho-groups, especially if the molecule is protonated (Fig. 7, right panel). Interestingly enough, positioning the azithromycin accross the hydrophilic/hydrophobic interface in the phospholipid monolayer occurred without misshaping of this interface. Thus, the drug truly inserts itself in the hydrophobic domain. This is in striking variance with what was found earlier for gentamicin, for which the projection of a polar moiety of the molecule (the 2',6' diamino sugar) towards the hydrophobic domain was accompanied by a profound misshaping of the interface, so that the molecule actually remained in an hydrophilic environment (Mingeot-Leclercq et al., 1989).

4. Discussion

The data presented in this contribution together with those reported in the companion paper (Van Bambeke et al., 1996a) establish that azithromycin, a dicationic macrolide, binds to and interacts with negatively charged phospholipid bilayers at acidic pH (5.4) and modifies several of their characteristics and properties. We present the hypothesis that this binding and interaction are responsible for the development of specific cytological alterations when the drug is added to cultured cells (Montenez, 1996) or administered to animals at large doses (Shepard et al., 1992) and consisting of a lysosomal phospholipidosis. Drug-induced phospholipidosis most often arises from the binding of the drug molecule to cell membranes, but this binding may involve very different types of interactions,

ranging from hydrophilic to hydrophobic ones (see gentamicin (Mingeot-Leclercq et al., 1989) and amiodarone (Chatelain et al., 1986), e.g., for contrasting examples). The present study strongly suggests that azithromycin is capable of establishing both types of interactions, because of its specific chemical structure. In cells, those interactions are expected to take place predominantly in lysosomes because this is where the drug preferentially accumulates (Gladue and Snider, 1990; Carlier et al., 1994) and because of the acidic pH of lysosomes [this pH is not modified from its normal value of ≈ 5.4, in spite of the extensive drug accumulation (Montenez, 1996)]. For these reasons, we have selected for our experiments a pH corresponding to that of lysosomes, and a lipid membrane composition mimicking that of biological membranes. Similar experiments could obviously be made in other conditions of pH or of membrane composition, with an aim at systematization, but the biological significance of the data would be questionable.

Interaction of azithromycin with negatively charged bilayers is clearly revealed by the effects seen in ^{31}P-NMR spectroscopy. At 33°C, control large multi-lamellar vesicles show a typical bilayer spectrum with a maximum at high field and a shoulder at low field. The general shape of this signal is similar to what was observed for biological membranes containing phospholipids and cholesterol (erythrocyte ghosts, chromaffin granule membranes; McLaughlin et al., 1975) or for lipids mixtures [phospholipids from *Escherichia coli* (Burnell et al., 1980a); mixture of phosphatidylethanolamine, phosphatidylcholine and cholesterol [3 : 1 : 2] (De Kruijff et al., 1979)]. In our system, the spectra of control liposomes were characterized, at low temperature, by a relatively small chemical shift anisotropy which decreased by temperature elevation. This chemical shift anisotropy is smaller than that usually reported for pure phospholipid dispersions [≈ 45 ppm for dipalmitoylphosphatidylcholine (McLaughlin et al., 1975)]. This could be due first, to the fact that the radius of the largest liposomes (≈ 2000 nm), which make up the bulk of large multi-lamellar vesicles population used here, puts the system at the limit where the chemical shift anisotropy can be influenced by vesicle size, as well as to a partial averaging of the chemical shift by tumbling, due to the relatively large dispersion of the size of the liposomes. A second and probably more important reason is the presence of cholesterol which is known to reduce the chemical shift anisotropy (McLaughlin et al., 1975). At high temperature, a sharp signal was detected at the isotropic shift value. The upsurge of this narrow signal is caused by the presence in the sample of a small proportion of more mobile, small vesicles that give a narrow signal at high temperature because of short correlation time (Burnell et al., 1980b). Raising the temperature of control liposomes also caused a reduction of chemical shift anisotropy, which reveals a greater mobility of the phosphorus atoms. This can be due to an increased mobility of the entire phospholipid molecule. However, the size of the large vesicles is such that the variation of the rate of overall isotropic diffusion over the temperature interval is probably not large enough to explain a decrease in the chemical shift anisotropy of ≈ 20%. Yet, the reduction of chemical shift anisotropy may also be contributed for by an increased mobility of the phosphate heads, which could occur independently and simultaneouly with the change of mobility of the lipid molecule.

The addition of the antibiotics produces two effects, viz. a severe reduction of the isotropic peak observed at high temperature and a decrease in the chemical shift anisotropy variation as a function of temperature. The first effect can be explained by specific interactions between drugs and phospholipids which reduce the mobility of the phosphorus atoms. The drugs might indeed interfere with the rates of lateral diffusion of the lipids reducing the effectiveness of the averaging process. Another explanation for the inhibition of the development of the sharp isotropic signal would be an increase in vesicle size produced by the antibiotic causing a reduction of tumbling rate of the vesicles and less efficient averaging of chemical shift anisotropy. This explanation is certainly applicable to the effect seen with gentamicin since this antibiotic is known to induce liposome aggregation (Mingeot-Leclercq et al., 1989) but it can be ruled out for azithromycin because we showed in the companion paper, and in comparison with gentamicin, that this antibiotic causes no aggregation or fusion of large unilamellar vesicles (Van Bambeke et al., 1996a). The two antibiotics also decrease the variation of chemical shift anisotropy developing over the temperature increase. Here, the increase in vesicle size caused by aggregation due to gentamicin may very slightly affect the chemical shift anisotropy averaging but another cause, viz. a reduction of the mobility of the phosphorus atoms, may also occur; both drugs may indeed reduce the motional properties of the lipids as such by decreasing their lateral diffusion rates. They may also interact directly with the hydrophilic part of the membrane and thereby reduce the phosphate head motion. If this is the case, the decreased mobility of the phosphorus atoms may also be interpreted as indicating a direct electrostatic interaction between the protonated amine(s) of the molecule and the adjacent phospho-groups. Both gentamicin and azithromycin primarily bind to negatively charged membranes by electrostatic interactions (Van Bambeke et al., 1996a), suggesting that a combination of the two mechanisms, viz. a decreased mobility of the entire lipid molecule and a reduction of the phosphate head motion, could be responsible for the decreased mobility of phosphorus atoms we observed.

In contrast to the observations obtained by ^{31}P-NMR, the influence of gentamicin and azithromycin on the fatty acyl chains must be very different, as judged from their contrasting behavior in the fluorescence polarization studies. Our experiments were made with liposomes containing a large proportion of cholesterol, which abolishes the

transition temperature by its particular ordering effect on the lipid matrix (for recent review, see Mouritsen and Jorgensen, 1994). This explains why the variation in polarization progresses as a linear function of the temperature. Yet, using probes, such as DPH and TMA-DPH, we are able to detect the difference in fluidity of the acyl chains between the deep zone of the bilayers and that close to the interface, as reported for biological membranes (Kitagawa et al. (1991); Zicha et al. (1993)). Davenport et al. (1985) have suggested that DPH is preferentially distributed at the center of the bilayer if its environment is chemically and physically heterogenous – which is probably the case for the liposomes used here. By contrast, TMA-DPH localizes in a region of the acyl chains close to the lipid/water interface, because of its positively charged moiety. Our data, therefore, indicates that azithromycin probably affects the fluidity of the acyl chains in that region. Yet, it does not affect the regions more deeply situated, and which are reached only by more lipophilic drugs, like DEH (Mingeot-Leclercq et al., 1989) or amiodarone (Chatelain et al., 1986). Thus, the insertion of azithromycin in the membrane must be deeper than that of gentamicin, but less pronounced than that of DEH or amiodarone. The position of azithromycin is, therefore, probably accross the interface, with some true penetration in the hydrophobic domain. The position of azithromycin is probably also similar to that of local anesthetics (tetracaine, procaine), which interact with the 12 first carbons of the lipids, as determined by ^{2}H-NMR but do not modify the polarization of DPH (Gutiérrez-Merino et al., 1989), or of daunorubicin, which is located in the same membrane domain as TMA-DPH (Ferrer-Montiel et al., 1988).

The conformational analysis essentially confirms that azithromycin, even when inserted in a monolayer of pure negatively charged phospholipids, is indeed capable of interacting both with the hydrophilic and the hydrophobic domains. It also shows that azithromycin is buoyant at the interface while protruding effectively into the adjacent hydrophobic domain. Using the same type of analysis, we found that gentamicin remains entirely in the hydrophilic domain whereas DEH deeply inserts itself in the hydrophobic domain, in accordance with the respective behavior deduced from the analysis of the biophysical data (for review, see Mingeot-Leclercq et al., 1995b).

In spite of this apparent coherence, the data generated by the conformational analysis raises a largely unanticipated issue, which is the fact that the uncharged and the charged forms of the molecule adopt not only very similar conformations, but also almost identical positions with respect to the interface. While this seems unplausible in view of the differences in water solubility of both forms, it must be remembered that the protonated form remains in any case sparingly soluble [limit of solubility in 0.1 M HCl $\approx$ 50 g/l (66 mM)]. The calculations actually reveal that, whatever the degree of protonation is, the molecule largely remains hydrophobic (hence its limited water solubility) and will, therefore, be attracted by lipophilic zones. The analysis of the virtual molecules shows that the methyl-group attached to the two ionizable functions largely masks their polar character, which greatly contributes to make the hydrophobic forces evenly distributed all around the molecule and minimizes its dipolar character. Moreover, the two amino-groups remain far apart from one another, preventing the formation of strong hydrophilic domain, even when protonated. It is only when a cluster of unprotected ionizable groups introduced in the 3-dimethyl-aminosugar moiety (virtual molecule DA-4) that a clearly polarized behavior is observed.

The conclusions of this part of the study would certainly benefit from the examination not only of virtual molecules, but also of actual derivatives of azithromycin, for which biophysical data could be experimentally obtained. This is the approach we followed in the past for gentamicin, and aminoglycosides in general, to ascertain its behavior in our conformational analysis approach and to further establish which parts of the molecule was responsible for the effects seen (see, e.g., Tulkens et al., 1990; Schanck et al., 1992). This development would, however, require to obtain homogenous series of azithromycin derivatives, which could not been undertaken in the context of the present study.

The position of azithromycin in the bilayer which we propose may explain a number of observations presented not only here and in the companion paper, but also in previous publications describing the interactions of this drug with constituents of mammalian tissues (see Foulds et al., 1990).

The lack of aggregating or fusogenic effect of azithromycin, compared with other cations, may be due not only to the masking of its cationic groups by the surrounding methyls first, but probably mainly by the actual position of the drug in the bilayer which will prevent it to efficiently bridge adjacent membranes (for a discussion of the process of fusion and aggregation induced by drugs, see Van Bambeke et al., 1996b). The drug anchored to the membrane will indeed behave largely like a monocation because of the large distance separating the $N_{3'}$ amino-group from the phosphate heads. The closeness of the N_{9a} amino-group from the interface will also prevent it to establish effective contacts with phospholipids of adjacent membranes.

The existence of strong hydrophobic interactions between phospholipids and azithromycin and its deep insertion in the bilayer may explain why azithromycin binds more to negatively charged membranes and, in spite of its reduced number of amino-groups (2 vs. 5), is as inhibitory as gentamicin towards the activity of phospholipase A_1 when tested for hydrolysis of phosphatidylcholine embedded in such membranes (see Van Bambeke et al., 1996a). The insertion of azithromycin in the bilayer may also suggest a mechanistic explanation for its slow release from lysosomes and cells in vivo and in cultured cells, which is

in contrast with what is observed with conventional monocationic macrolides [erythromycin (Martin et al., 1985) and roxithromycin (Carlier et al., 1987)], for instance, are released from cells almost at the same rate as they are accumulated). Interestingly enough, azithromycin release becomes increasingly slower upon repeated duration of administrations. Because repeated administration is probably more susceptible to cause phospholipid accumulation than a single dose (see discussion in Van Bambeke et al., 1996a; Montenez, 1996), it is tempting to speculate that the drug becomes sequestered with the slowly accumulated phospholipids, giving rise to an increasingly larger 'deep compartment'. We lack, however, of kinetics studies of azithromycin binding and release from membrane layers to further substantiate this hypothesis.

Another issue is whether the mode of interaction with phospholipid layers proposed for azithromycin is compatible with its proposed mode of accumulation in lysosomes, viz., proton trapping. This mechanism (reviewed in general terms by De Duve et al., 1974; see also Carlier et al., 1987, for macrolides in general, and Carlier et al., 1994, for azithromycin) assumes that the unprotonated form is more lipophilic, and, therefore, diffuses considerably more quickly than the protonated form. Yet, our analysis fails to disclose marked differences between the two forms of the drug in this respect. However, as pointed out above, the present studies, including the conformational analysis, analyze a situation at equilibrium and may not necessarily be predictive of the kinetic behavior of the two forms of azithromycin when encountering membrane bilayers.

In spite of these many remaining uncertainties, the present investigations may nevertheless represent an appropriate set of background data explaining key features in azithromycin interactions with phospholipids, shedding new light on its unusual pharmacokinetic properties as well as its potential effects on phospholipid catabolism and lysosomal physiology. These approaches may also be useful for the prospective evaluation of other derivatives with large tissue accumulation in this family of antibiotics.

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