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Interactions of Macrolide Antibiotics (Erythromycin A, Roxithromycin, Erythromycylamine [Dirithromycin], and Azithromycin) with Phospholipids: Computer-Aided Conformational Analysis and Studies on Acellular and Cell Culture Models

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The potential of 14/15 membered macrolides to cause phospholipidosis has been prospectively assessed, and structure–effects examined, using combined experimental and conformational approaches. Biochemical studies demonstrated drug binding to phosphatidylinositol-containing liposomes and inhibition of the activity of lysosomal phospholipase A_1 toward phosphatidylcholine included in the bilayer, in close correlation with the number of cationic groups carried by the drugs (erythromycin A ≤ roxithromycin < erythromycylamine ≤ azithromycin). In cultured cells (fibroblasts), phospholipidosis (affecting all major phospholipids except sphingomyelin) was observed after 3 days with the following ranking: erythromycin A ≤ roxithromycin < erythromycylamine < azithromycin (roxithromycin could, however, not be studied in detail due to intrinsic toxicity). The difference between erythromycylamine and azithromycin was accounted for by the lower cellular accumulation of erythromycylamine. In parallel, based on a methodology developed and validated to study drug–membrane interactions, the conformational analyses revealed that erythromycin A, roxithromycin, erythromycylamine, and azithromycin penetrate into the hydrophobic domain of a phosphatidylinositol monolayer through their desosamine and cladinose moieties, whereas their macrocycle is found close to the interface. This position allows the aminogroups carried by the macrocycle of the diaminated macrolides (erythromycylamine and azithromycin) to come into close contact with the negatively charged phosphogroup of phosphatidylinositol, whereas the amine located on the C-3 of the desosamine, common to all four drugs, is located at a greater distance from this phosphogroup. Our study suggests that all macrolides have the potential to cause phospholipidosis but that this effect is modulated by toxicodynamic and toxicokinetic parameters related to the drug structure and mainly to their cationic character. © 1999 Academic Press

Macrolide antibiotics (erythromycin A and related drugs) are used extensively worldwide due to their perception as safe and effective antibiotics against a broad range of infections caused by Gram (+) organisms as well as a series of intracellular pathogens (*Legionella p., Chlamydia spp. . . .*) for which there is only a limited number of alternative treatments. As weak organic bases, macrolides accumulate in the lysosomes of eucaryotic cells (Carlier *et al.,* 1987, 1994; Tulkens, 1991 for review), raising potential concerns for toxicity. We indeed demonstrated that azithromycin, a dicationic macrolide with a high degree of accumulation and retention in tissues (Foulds *et al.,* 1990), causes a typical lysosomal phospholipidosis in cultured fibroblasts (Van Bambeke *et al.,* 1996, 1998). This type of alteration has been previously reported for the so-called amphiphilic cationic drugs (Kodavanti and Mehendale, 1990) and for aminoglycoside antibiotics (Aubert-Tulkens *et al.,* 1979). These drugs were shown to bind to negatively charged phospholipid bilayers at acidic pH (Laurent *et al.,* 1982) and to inhibit the activities of lysosomal phospholipases by neutralizing the surface negative charges (Chung *et al.,* 1985) required by these enzymes for optimal activity (Mingeot-Leclercq *et al.,* 1988). Since azithromycin was also shown to interact with acidic phospholipids and to inhibit lysosomal phospholipases (Van Bambeke *et al.,* 1996, Montenez *et al.,* 1996), we have examined in a prospective way the potential of other macrolides to also induce a phospholipidosis. By combining a computer-aided conformational analysis with a series of experimental approaches using acellular models and cultured cells, we present here a detailed molecular description of the interactions of macrolides with negatively charged phospholipids and an insight on their potential effects toward the lysosomal catabolism of phospholipids. For this purpose, we have studied

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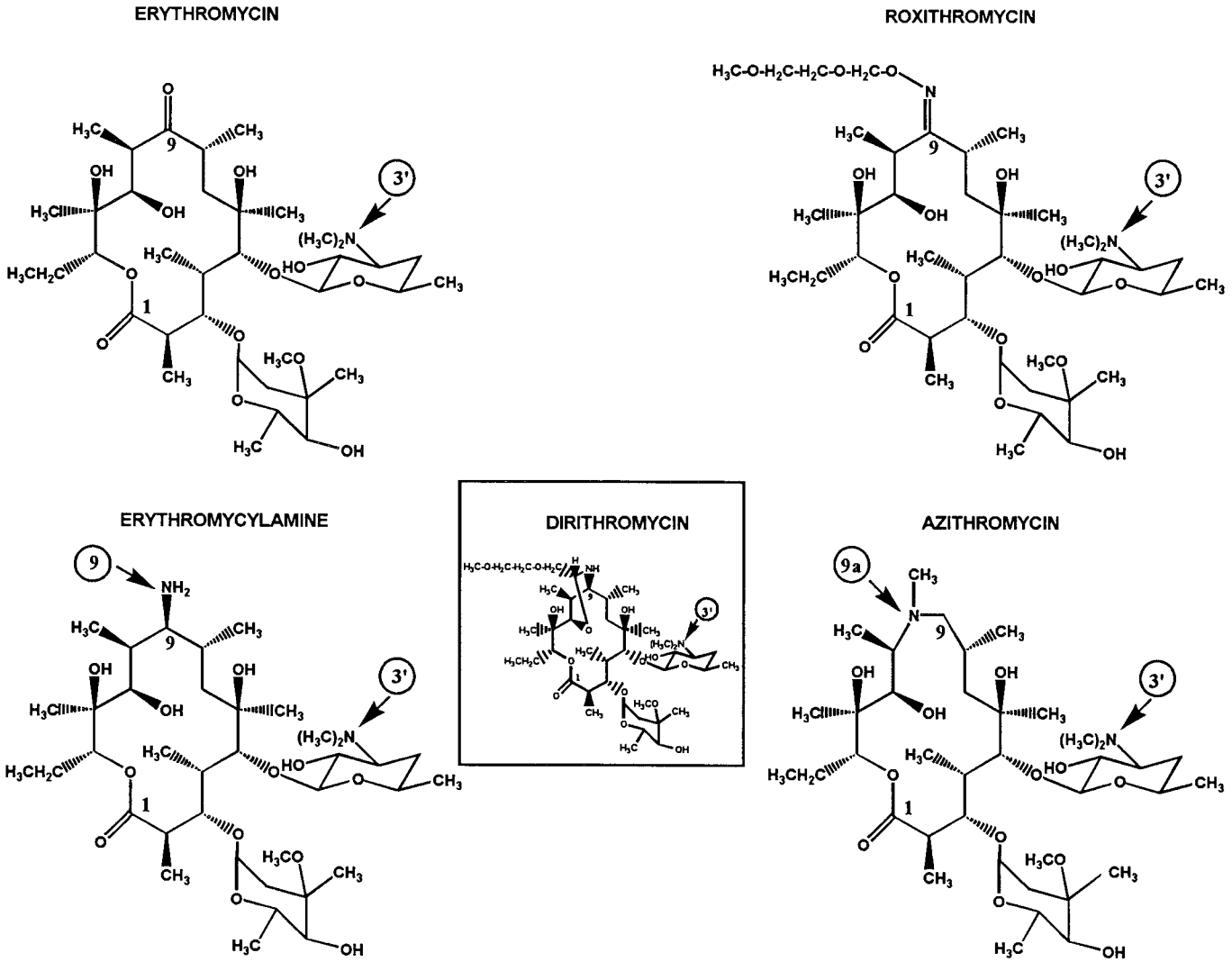

FIG. 1. Structural formulae of the macrolides investigated. The circled numbers point to the aminofunctions central to the present study, the pK_as of which all lie in a narrow range (8.1 to 9.5) so that they are fully ionized at the lysosomal pH (≈5). The numbering of the atoms in the macrocycle shown here follows the historical convention adopted for erythromycin (Wiley, *et al.*, 1957; Harris, *et al.*, 1965) and counts them counterclockwise starting from the carbonyl (CO) adjacent to the lactonic oxygen (the IUPAC nomenclature used for systematic chemical names would count atoms clockwise from the lactonic oxygen). All molecules are characterized by a lactonic macrocycle substituted in Position 5 (*trans*) by an *aminated* sugar (*β*-D-desosamine [3′,4′,6′-trideoxy-3′-(dimethylamino)-*β*-D-xylo-hexopyranosyl]). Erythromycin A and roxithromycin (9-[*O*-(2-methoxyethoxy)methyl]oxime erythromycin A; [Chantot *et al.*, 1986]) carry no basic function on their macrocycle (the *N* atom substituting C-9 in roxithromycin is not ionizable in a physiological pH range, being part of a *N*-oxime function); these drugs are therefore monobasic. Erythromycylamine ((9-*S*)-9-deoxy-9-amino-erythromycin A; [Massey *et al.*, 1970]) and azithromycin (9-deoxo-9a-methyl-9a-azahomoerythromycin A; [Bright, *et al.*, 1988; Djokic, *et al.*, 1988]) both possess a basic aminofunction in their macrocycle (a primary amine substituting C-9 in erythromycylamine and an endocyclic tertiary amine in azithromycin (Position 9a; this position is also referred to as Position 10 in some nomenclatures). Azithromycin and erythromycylamine are therefore dibasic. Erythromycylamine is commercialized as its prodrug dirithromycin (inset; (*R*)-methoxyethoxymethyl-9-(*S*)-*N*,11-*O*-oxazine-erythromycylamine).

two monocationic macrolides (erythromycin A and roxithromycin) in comparison with azithromycin and another dicationic macrolide, erythromycylamine. These derivatives, selected to allow to draw structure side-effects considerations (as was done successfully in the past for aminoglycosides [Brasseur *et al.*, 1984; Mingeot-Leclercq *et al.*, 1991; Kotretsou *et al.*, 1995]), show a similar overall structure, but with some specific structural differences in comparison with azithromycin (Fig. 1). All four drugs indeed carry an aminofunction on one of the two sugars (desosamine) attached to the macrocycle. Erythromycylamine and azithromycin both possess an additional aminofunction on their macrocycle at almost the same position but of a different nature (a primary amine attached to the C-9 in erythromycylamine and an endocyclic tertiary amine in azithromycin). In comparison, erythromycin A and roxithromycin are both monocationic, but the latter possesses a rather bulky side chain attached to C-9 in place of the ketogroup of erythromycin A. All four drugs are in current clinical use.

Erythromycin A represents the first macrolide introduced in the clinics and is still widely used under a large number of galenic forms. Roxithromycin (Chantot *et al.*, 1986) is one of the first acid-stable macrolides developed for human use and is largely used in Europe and South America. Erythromycylamine, originally described by Massey *et al.* (1970), is used nowadays as its prodrug dirithromycin (Luger and Maier, 1979; Counter *et al.*, 1991) in several countries. Azithromycin (Bright *et al.*, 1988; Djokic *et al.*, 1988) is now used worldwide as an effective drug for most indications of macrolides, including long-term prophylaxis of intracellular infections in AIDS patients.

MATERIALS AND METHODS

Computer-Aided Conformational Analysis

This study was made for all four antibiotics as well as for the prodrug form of erythromycylamine, dirithromycin. We used data obtained by X-ray crystallography to calculate the initial conformers of each drug since it makes it easier to obtain an energetically stable conformation during the optimization procedure (Brasseur *et al.*, 1982). For erythromycin A and erythromycylamine, we used the coordinates of the parent molecules clarithromycin (6-dehydroxy-6-methoxy-erythromycin A) and *N*-(2-ethyl-1-butyl)erythromycylamine (Kirst *et al.*, 1990), respectively, which were directly available from the Cambridge Structural Database (Cambridge Crystallographic Data Center, 12 Union Road, Cambridge, England) as refcodes WANNUU and JICYUP, respectively. The data were put in Hyperchem 5.0 software compatible format and modified to obtain erythromycin A, and 9 (*S*)-erythromycylamine and dirithromycin, respectively. For azithromycin and roxithromycin, we used the *inverted* values along one axis of the stereostructures stored in the Cambridge Structural Database as refcodes GEGJAD and FUXYOM, respectively (it appeared indeed that the values contained in the Database yielded conformations systematically opposite to those of the genuine compounds; this problem, which relates to a technical mistake, was kindly pointed out to us by Dr. H.A. Kirst, Eli Lilly Research Laboratories, Greenfield, Indiana; see Sheldrick *et al.*, 1995 and Montenez *et al.*, 1996 for an explanation concerning azithromycin; no explanation has been received so far concerning roxithromycin). Macrolides were considered both under their protonated and unprotonated forms. The first one corresponds to that encountered in acidic lysosomal environment where macrolides are accumulated (pH $\approx$ 5) since the pK_a's of all groups are comprised between 8.1 and 9.5. It was modeled by adding hydrogen atoms on each of the amino functions and by giving 1 (erythromycin A, roxithromycin) or 2 (erythromycylamine, dirithromycin, azithromycin) positive charges to the whole molecule. The distribution of these total charges on the individual atoms was performed using the CNDO procedure of Hyperchem 5.0. The method used for calculation and orientation of the isolated molecules at a lipid/water interface and for construction of mixed monolayers drug:phosphatidylinositol has been developed and reviewed in Brasseur (1990). It takes into account the interface properties with the dielectric constant increasing linearly from 3 to 30 to mimic a biological membrane along an axis perpendicular to the putative interface. The total conformational energy of the isolated molecule was calculated as the sum of London-van der Waals' energy of interaction, the electrostatic interaction, the potential energy of rotation of torsional angles, and the transfer energy of atoms or groups of atoms from a hydrophobic to an hydrophilic phase (Brasseur, 1990). The values of the transfer energies (E_{tr}) used were similar to those determined experimentally (Brasseur, 1990). The hydrophobic (E^{tr}_{pho}) and the hydrophilic (E^{tr}_{phi}) transfer energies of a given conformation are the sum of all transfer energy changes associated with the transfer of an atom *i* from one to the other phase (Brasseur *et al.*, 1986; Brasseur, 1990). At each step of the minimization procedure, the molecule was oriented with the line joining the hydrophilic and hydrophobic centers perpendicular to the interface. The hydrophilic center ($\vec{C}_{phi}$) was defined as $\vec{C}_{phi} = \Sigma E^{tr}_{phi} \vec{r}_i / \Sigma E^{phi}_{tr}$ in which $\vec{r}_i$ are the coordinates of the *i*th hydrophilic atom. The hydrophobic center located in the hydrocarbon domain was defined by a similar equation, except that the negative transfer energies associated to the hydrophobic atoms are taken into account. The hydrophobic/hydrophilic balance is defined as $\log (\Sigma E^{tr}_{pho} / \Sigma E^{tr}_{phi})$. The distance between the hydrophobic and the hydrophilic centers is defined as $|\vec{C}_{pho} - \vec{C}_{phi}|$. The energy of interaction between the drug and lipid molecules is the sum of the London-van der Waals' energy of interaction, the electrostatic interaction, and the transfer energy of atoms or groups of atoms from a hydrophobic to an hydrophilic phase (Brasseur, 1990). The method used has proven useful for the description of the interaction of several other drugs with membranes (aminoglycosides, adriamycin, ethidium bromide, antimycotics, propranolol, various alcohols and ionophores [Brasseur, 1990], and azithromycin [Montenez *et al.*, 1996]). A satisfactory agreement between the conclusions obtained by the application of the methodology used here and those obtained from neutron diffraction, X-ray diffraction, and polarized infra-red spectroscopy studies has been reported for lipids (Brasseur *et al.*, 1981), ionophores (lasalocid A; Brasseur *et al.*, 1982), and peptides (Brasseur *et al.*, 1990). Detailed information on computer programs and on their characteristics is available from their author (R.B., E-mail: brasseur.r@fsagx.ac.be).

Experimental Studies

Antibiotics. Erythromycin A was supplied by Abbott S.A., Louvain-La-Neuve, Belgium as the lactobionate salt (powder) used for parenteral administration to humans (Erythrocine). The other antibiotics were supplied as base for investigational purposes (roxithromycin by Erfa S.A., Brussels, Belgium [on behalf of Hoechst-Marion-Roussel S.A., Brussels, Belgium]; azithromycin by Pfizer Mack Pharm. Dev., Illerstissen, Germany; and erythromycylamine, as its 9-*N*, 11-*O*-oxazine prodrug derivative [dirithromycin], by E. Lilly & Co, Indianapolis, IN). Erythromycin A (as lactobionate salt) was readily water soluble and was therefore directly dissolved in the appropriate buffer or in the cell culture medium at the desired concentrations and used as such. The other antibiotics, supplied as bases, are not readily water soluble and the following procedures were therefore adopted. For experiments with liposomes, for which we wanted to avoid the presence of residual organic solvent that could affect the structural organization of the lipids, the free bases were first dissolved in 0.1 N HCl at a concentration of 25 mg/ml, brought to pH 5.4 with NaOH, and then diluted to the desired concentrations in 40 mM acetate buffer pH 5.4. For cell culture experiments, roxithromycin and dirithromycin were first dissolved in ethanol, and azithromycin was dissolved in dimethylsulfoxide, at a concentration of 25 mg/ml. This solution was thereafter diluted at least 100-fold in the final culture medium (we checked on the basis of trypan blue exclusion tests that the residual amounts of ethanol or dimethylsulfoxide did not affect cell viability). For erythromycylamine, all final solutions were left for 3 h at room temperature before use to allow more than 90% hydrolysis of dirithromycin to erythromycylamine ($t_{1/2} \approx 40$ min [Kirst *et al.*, 1995]) prior to inclusion in the cell culture medium or in the biochemical assay mixtures. For all drugs, fresh solutions were prepared for each series of experiments. All concentrations refer to the free base of each drug.

Other products. Egg yolk phosphatidylcholine and wheat germ phosphatidylinositol (grade 1 products) were obtained from Lipid Products (Nr Redhill, U.K.); bovine brain sphingomyelin and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO); radiolabeled phosphatidylcholine (1-palmitoyl-2 [1-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine; 52 mCi/mmol) was obtained from Amersham International plc (Amersham, U.K.). Other reagents were obtained from E. Merck AG (Darmstadt, Germany) and were of analytical grade.

Assay of macrolides. Because of lack of a radiolabeled form of erythromycylamine, all four drugs were assayed by a microbiological technique for sake of consistency throughout the present study. We used the disc-plate method with *Bacillus subtilis* as test organism (Grove and Randall, 1955). In brief, paper disks (0.25 inch diameter, Becton Dickinson, Cockeysville, MD) soaked with 20 μl of cell lysates (or appropriate standards) were placed on the surface of a Neomycin Assay agar Antibiotic medium 11 (Becton Dickinson)

inoculated with sporulated bacteria and incubated for 24 h at 37°C. Antibiotic concentration in samples was then calculated by comparing the diameter of growth inhibition to that of the standards using linear regression and intrapolation. The method had a lower limit of detection of 1 mg/liter, a coefficient of variation of 4.5% (calculated on 18 samples made in triplicate), and an interday repeatability of a same standard of 1.8% ($n = 4$, each assay being made in triplicate).

Preparation of liposomes. Small unilamellar vesicles (SUV) made of cholesterol, phosphatidylcholine, sphingomyelin, and phosphatidylinositol (molar ratio of 5.5:4:5:2) were prepared by sonication in 40 mM acetate buffer at pH 5.4. As described previously (Mingeot-Leclercq *et al.,* 1988), a lipid film was prepared by solvent evaporation in a round-bottomed flask, hydrated with the buffer for 1 h in a nitrogen atmosphere, and thereafter submitted to five cycles of 2 min sonication at 40 W in an ice bath. The final suspension was centrifuged at 200*g* during 10 min to eliminate the titanium particles released from the ultrasonic probe. Each preparation was used within 2 days.

Determination of phospholipase A_1 activity (phospholipid 1-deacylase, EC 3.1.1.32). Assay was made toward 1-palmitoyl,2-[1-^{14}C]oleoylphosphatidylcholine included in liposomes (140 mCi/mol of phosphatidylcholine; total lipid concentration, 15.7 mM) using the method described in Laurent *et al.* (1982). The enzyme source was a soluble extract of purified rat liver lysosomes. Activity was defined as the net amount of labeled substrate converted into β-lysophosphatidylcholine. The latter was separated from phosphatidylcholine by thin-layer chromatography using chloroform:methanol:acetic acid:water (25:15:8:4) as mobile phase, and the corresponding parts of the gel were cut and used for radioactivity determination using scintillation counting.

Binding of macrolides to phospholipids. This was investigated by equilibrium dialysis toward liposomes using a Dianorm apparatus (Dianorm Geräte, München, Germany), consisting of sets of twin cells with a 250 μl volume (filled with 200 μl only to allow for a permanent mixing upon cell rotation) separated by a Diachema flat dialysis membrane (MW cutoff 5000). Dialysis was performed for 5 h at 37°C under constant rotation at 4 rpm (Van Bambeke *et al.,* 1996). Determination of the free antibiotic concentration was made by microbiological assay (see above) of the content of the liposome-free chamber; the concentration of antibiotic in the liposome-containing chamber (free + bound) was then calculated from the difference between the amount of drug detected in the liposome-free chamber and the total amount of drug introduced in the system (this method had been validated previously for azithromycin with respect to both the time needed to reach equilibrium and drug recovery [Van Bambeke *et al.,* 1996]). The initial drug concentration was set up at 120 μM in all cases, and the composition and the total lipid concentration of liposomes were the same as that used for the assay of phospholipase A_1.

Cell culture and collection. Primary cultures of rat fibroblasts were obtained by trypsinization (0.25% trypsin in Ca^{2+} and Mg^{2+}-free Hanks' solution at 37°C; Tulkens *et al.,* 1974) of eviscerated 18-day-old embryos of pregnant rats of (Wistar strain; Animalerie facultaire of the Université Catholique de Louvain, Brussels, Belgium). Cells were collected by centrifugation (200*g*; 20 min) and washed two times with DMEM medium with 10% fetal calf serum. Cultures were seeded with 10^5 cells/cm^2 of growing surface. After 7 days, monolayers were disrupted and cells were detached with 0.1% trypsin in PBS phosphate-buffered saline (0.15 M NaCl, 2.7 mM KCl, and 3 mM Na_2HPO_4–KH_2PO_4; pH 7.4) and subcultured at a density of about 5×10^4 cells/cm^2. Cultures were performed in a 5% CO_2 atmosphere using a DMEM medium supplemented with 10% fetal calf serum. Cells were used at the first subculture once reaching confluency (4 to 7 days). At the end of the incubation period with the macrolides, the cell sheets were washed four times with ice-cold NaCl 0.9%, collected by scraping in NaCl 0.9%, and subjected to vigorous shaking and brief sonication to achieve complete disruption. The cells' homogenates were stored at −20°C until used for microbiological or biochemical assays. Total cell protein was assayed by the Lowry *et al.*'s (1951) technique using serum albumin as standard.

Determination of cell integrity during incubation with the antibiotics. This was assessed by the measurement of the release of the cytosolic enzyme, lactate dehydrogenase (EC 1.1.1.27), in the culture medium. Activity was measured on the culture medium using the method of Vassault (1987) after low-speed centrifugation (200*g*; 10 min) to remove intact cells, and corrected for by the activity found in a sample of the same culture medium unexposed to cells (serum enzyme; this correction corresponded to approximately $\frac{2}{3}$ of the activity measured in the medium exposed to cells). The activity of the medium was then compared to the activity observed in the cells harvested and disrupted as described above, and the release was expressed as the percentage of the total activity measured in the whole cell culture (cells + medium).

Determination of the cell antibiotic content and calculation of the apparent drug accumulation. The cell drug content was expressed as nmol of drug per mg of cell protein, using appropriate calibration of the microbiological assay (see above). To establish whether all cell-associated drug was quantitatively detected, we compared the results obtained in the present study for erythromycin A, roxithromycin, and azithromycin with historical data from earlier studies of our laboratory in which antibiotic accumulation drugs had been studied using ^{14}C-labeled drugs (results partially reported in Carlier *et al.,* 1987, 1994). No meaningful difference was observed between the two sets of data, indicating that the procedure used was effective and also strongly suggesting that no major drug metabolism occurred in the cells used (or that such metabolites are not retained by cells), since all known metabolites from the macrolides investigated are devoid of antimicrobial activity. To better emphasize the fact that cells accumulate macrolides to a large extent, we also calculated the ratio of the apparent cellular drug concentration to its extracellular value (a widely used parameter to assess and compare antibiotics accumulating in cells [Tulkens, 1991]). This ratio was calculated assuming that 1 mg of cell protein corresponds to a cell volume of 5 μl (a figure close to this value has been obtained experimentally for cultured fibroblasts in our laboratory [Tulkens and Trouet, 1978]). It must however be remembered that the macrolides are not homogeneously distributed within the cell (Carlier *et al.,* 1987, 1994). This ratio of cellular to extracellular concentrations is therefore referred to as an *apparent* accumulation.

Determination and analysis of cell phospholipids. Total phospholipids were extracted and assayed as described earlier (Laurent *et al.,* 1982) (intraday coefficient of variation: 1.5% [$n = 20$]). Individual phospholipids were separated by thin-layer chromatography on silica gel plates (E. Merck) with two successive developments with chloroform:methanol:acetic acid:water (65:50:1:4 v/v). The plates were then exposed to iodine vapors and the spots were identified by comparison with known standards run in parallel. The silica gel was scraped from the plates and phosphorus was assayed by the method of Bartlett (1959) after mineralization in 60% perchloric acid at 210°C for 90 min. Recovery of phosphorus from the original samples varied between 80 and 85%.

Statistical analyses. Unless stated otherwise, results presented are the means ± SD of three independent experiments. When appropriate, a two-way anaylsis of variance (ANOVA) was performed to compare treatments with different drug concentrations as a function of time. Student's *t* test was applied when comparing data from one experimental point with the corresponding control or with another set of appropriate experimental values.

RESULTS

Conformational Analyses

In the first part of this analysis, we determined the conformation of the five molecules at an hydrophilic/hydrophobic interface. Images are presented in Fig. 2 and the numeric data concerning the key conformational parameters are listed in Table 1 (the data on azithromycin have already been published in Montenez *et al.* [1996], but are reproduced here for sake of comparison). Two key observations were made at this stage. First, the conformation of all five molecules was not markedly influenced by the protonation of their aminogroups. Second, it appeared that the positions and orientations of all four antibi-

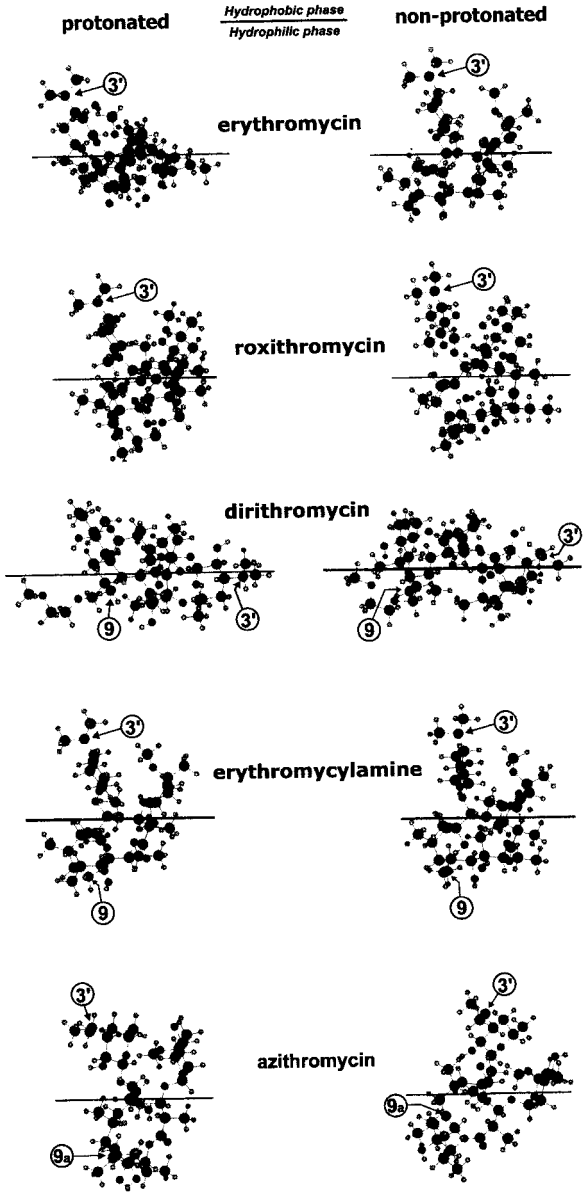

FIG. 2. Most probable conformers of the five macrolides studied (balls and sticks models) under their nonprotonated (left) and protonated (right) forms, at a lipid–water interface (indicated by the horizontal bar, the lipid phase being above). The positions of the aminofunctions are pointed by the numbered arrows (3′ denotes the tertiary amine carried by the β-D-desosamine [all drugs], 9 the primary amine in C-9 of the macrocyle of erythromycylamine, [and the corresponding N in its prodrug dirithromycin], and 9a the endocyclic tertiary amine of azithromycin; see Fig. 1 for structural formulae).

otics (thus excluding the prodrug dirithromycin) at the hydrophobic–hydrophilic interface were very much akin and minimally influenced by the number of aminogroups carried by the molecules. Thus, the macrocycle of each antibiotic was largely located in the hydrophilic zone, probably in relation with the presence of the three hydroxyl functions at C-6, C-11, and C-12 and of the lactonic function (-O-CO-). Accordingly, the 9 ketogroup of erythromycin A, the *N*-oxime side-chain of roxithromycin, the *N*-9 primary amine of erythromycylamine (attached to its C-9), and the tertiary endocyclic amine [*N*-9a] of azithromycin were all in the hydrophilic region. In contrast, the cladinose and the β-D-desosamine were systematically located in the hydrophobic zone for all four antibiotics. In contrast, and as also shown in Fig. 2, the substitution of the *N*-9 amino and of the *O*-11 hydroxylfunctions of erythromycylamine by the 2-(2-methoxyethoxy)
ethylidene moiety [dirithromycin] caused a marked clockwise rotation of the molecule bringing the *N*-3′ tertiary amine of the desosamine to the interface, while the side-chain itself remained in the aqueous phase.

In the second part of the analysis, we constructed mixed monolayers of drugs and phosphatidylinositol. As shown in Fig. 3 for the protonated molecules, these mixed monolayers could be constructed without grossly disturbing the organization of the lipids. This form of drug–lipid assembly was not very different for the unprotonated molecules (data not shown; note that the interaction energy of both forms is similar [Table 1]) and all five molecules could be surrounded by almost the same number of phosphatidylinositol molecules (six to seven; see Table 1) whether protonated or not. The orientation adopted by azithromycin and erythromycin A allowed the aminogroup present on their macrocycles (the endocyclic *N*-9a in azithromycin, and the *N* attached to C-9 in erythromycylamine) to be located at a short distance from the negatively charged phosphogroup of phosphatidylinositol, so that it could easily interact and neutralize it (a similar localization is also observed for the *N* atom of the *N*-oxime side-chain of roxithromycin, but this *N* is nonionizable). In contrast, the aminogroup carried by the desosamine was located at a greater distance from the negatively charged phosphogroup of the phospholipid, and therefore not expected to interact significantly with it (note, however, that the *N*-3′ amino function of dirithromycin [the prodrug of erythromycylamine] is located much closer to the hydrophilic domain because of the reorientation of the molecule brought by the presence of the methoxyethoxyethylidene side chain).

Experimental Studies

In these studies, only erythromycin A, roxithromycin, erythromycylamine, and azithromycin were used for reasons of chemical instability of dirithromycin (see Kirst, *et al.*, 1995 for the rate of conversion of dirithromycin into erythromycylamine in aqueous media).

Inhibition of lysosomal phospholipase A_1 in acellular systems. Because previous studies had shown that azithromycin inhibits the activity of lysosomal phospholipases (toward phosphatidylcholine, a major membraneous phospholipid, included in negatively charged bilayers), we examined the behavior of the other drugs in this context. The study was restricted to phospholipase A_1, because deacylation of phosphatidylcholine in Position 1 represents the major route of degradation of this phospholipid by lysosomal extracts (Laurent *et al.*, 1982). An

TABLE 1
Key Conformational Parameters of the Macrolides as Isolated Molecules at a Lipid–Water Interface and in Assembly with Phosphatidylinositol

	Isolated drug				Drug in mixed monolayer	
	Transfer energy (kcal/mol)		Hydrophobic/hydrophilic balance	Distance between centers (Å)	No. of lipid molecules in the complex	Energy of interaction (kcal/mol)
	Hydrophobic	Hydrophilic				
Erythromycin A						
Nonprotonated	−142.5	45.0	0.501	0.60	7	−167.1
Protonated	−142.5	46.0	0.491	0.48	7	−132.8
Roxithromycin						
Nonprotonated	−159.6	53.7	0.473	0.77	6	−186.8
Protonated	−159.6	54.7	0.465	0.65	6	−180.1
Dirithromycin						
Nonprotonated	−163.1	50.9	0.506	0.36	7	−166.4
Protonated	−163.1	52.9	0.489	0.43	7	−191.2
Erythromycylamine						
Nonprotonated	−144.0	47.3	0.484	0.78	7	−191.4
Protonated	−144.0	49.3	0.465	0.71	6	−159.6
Azithromycin						
Nonprotonated	−150.2	45.2	0.521	0.46	6	−184.8
Protonated	−150.2	47.3	0.502	0.39	6	−196.2

important feature in our *in vitro* model is the presence of phosphatidylinositol in the bilayer, which needs to be present to 18% of total phospholipids or more, together with cholesterol and sphingomyelin, to obtain maximal activity of the enzyme (Mingeot-Leclercq *et al.*, 1988).

Figure 4 shows that all four macrolides inhibit the activity of phospholipase A_1, down to a residual value of approximately 25% of that of controls when the drug concentration reaches a value of 400 μM. Yet, at lower concentrations, the dicationic macrolides (erythromycylamine, azithromycin) appear considerably more inhibitory than the monocationic ones (erythromycin A, roxithromycin), with a three- to fourfold difference in IC_{50}s (concentrations needed to achieve 50% inhibition, as determined by graphical intrapolation) between these two groups.

Binding of macrolides to negatively charged liposomes. Our previous studies have indicated that the inhibition of the activity of lysosomal phospholipases by aminated drugs in our experimental system (viz. azithromycin [Van Bambeke *et al.*, 1996], and the aminoglycoside antibiotics [Laurent *et al.*, 1982]) is related to their binding to the liposomes. We therefore examined directly the ability of all four macrolides to effectively bind to the lipid vesicles used for the inhibition studies. This binding was studied by equilibrium dialysis, setting up the drug concentration at 120 μM, i.e., at a value at which inhibition is almost maximal for azithromycin and erythromycylamine but submaximal for erythromycin A and roxithromycin. Figure 5 shows that approximately 18% of erythromycin and roxithromycin was bound to liposomes, whereas the binding of azithromycin and erythromycylamine reached 35 and 50%, respectively (the difference between these two drugs being significant at $p < 0.01$).

Studies with cultured cells. In preliminary experiments, we checked that cells withstood incubation with each of the macrolides at large concentrations for up to 3 days, using as criteria the release of lactate dehydrogenase in the medium (twice the release observed in control cells, the latter being <10% of the total enzyme activity present in the whole culture system). This led us to choose 50 mg/liter as a maximum value for roxithromycin and azithromycin and 250 mg/liter for erythromycin A and erythromycylamine. We then measured the level of drug accumulation and the cell content in phospholipids after 3 days of incubation. Results are shown in Table 2. We first noted that uptake was not saturable in the 0 to 50 mg/liter range since the cell drug content was five times larger in cells incubated with 50 mg/liter vs those incubated at 10 mg/liter. Second, major differences in accumulation were observed between drugs (erythromycin A < roxithromycin ≈ erythromycylamine < azithromycin). Third, erythromycin A, erythromycylamine, and azithromycin caused significant increases in cell phospholipids, although the minimal extracellular concentration necessary to induce this effect was markedly different (erythromycin A > erythromycylamine > azithromycin). Roxithromycin at 250 mg/liter caused an ≈50% increase in total phospholipid contents, but these data are not included in Table 2 since the viability of the cells was considered as uncertain at that drug concentration (threefold increase in the release of lactate dehydrogenase over control). To further characterize the influence of erythromycin A, erythromycylamine, and azithromycin on the change in cell phospholipid content, we investigated the influence of the time incubation at increasing drug concentrations (Fig. 6). For all three drugs, a steady increase was observed at each of the largest concentrations investigated (250 mg/liter for erythromycin A and erythromycylamine; 50 mg/

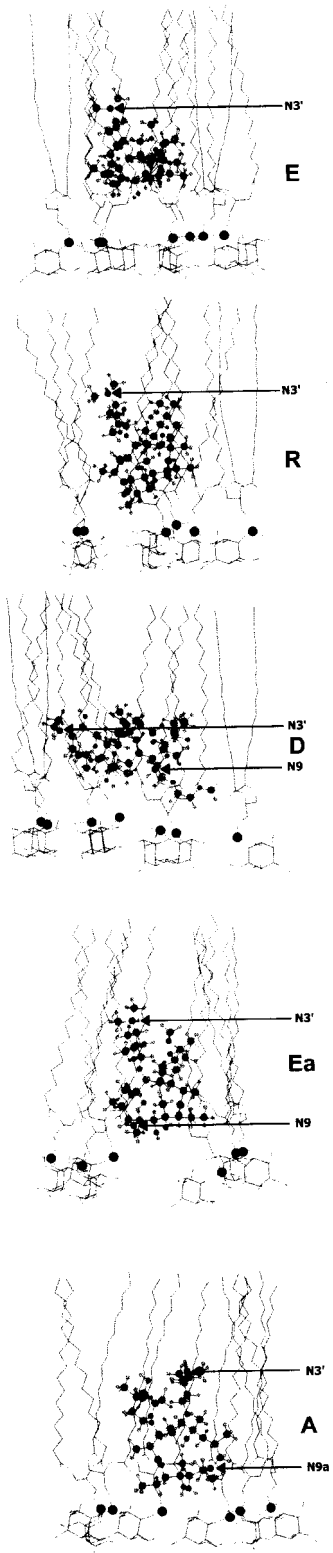

FIG. 3. Mixed monolayers of phosphatidylinositol and macrolides (E, erythromycin A; R, roxithromycin; D, dirithromycin, Ea, erythromycylamine; A, azithromycin) under their protonated form. The orientation and mode of representation of the drugs are essentially those used in Fig. 2. The phospholipid molecules are represented as skeleton only, except for their phosphorus atom. The position of the drug ionizable *N* is shown by the horizontal bars.

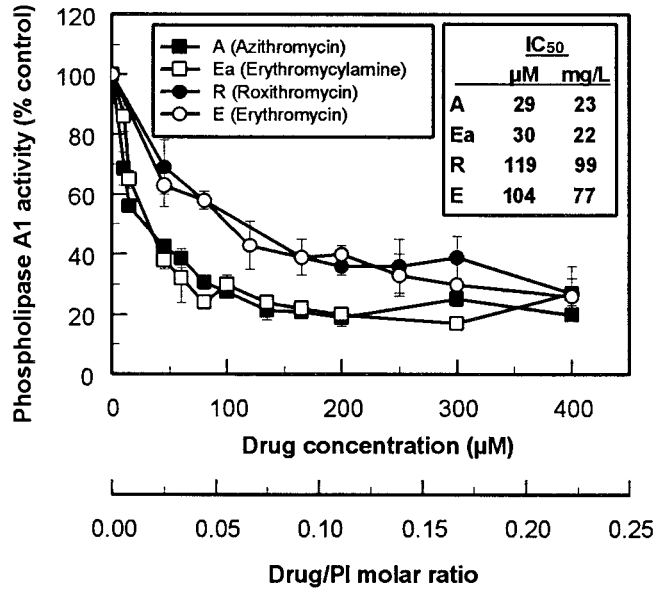

FIG. 4. Inhibition of the activity of lysosomal phospholipase A_1 by macrolides toward phosphatidylcholine included in negatively charged liposomes (cholesterol:phosphatidylcholine:sphingomyelin:phosphatidylinositol; molar ratio 5.5:4:5:2; final concentration in total lipids, 15.7 mM). The ordinate shows the activity as percent of the value recorded in the absence of antibiotic. The abcissa gives the drug concentration (main scale), together with the molar ratio between the drugs and phosphatidylinositol (lower scale). Results are the means ± SD of three independent determinations. The inset shows the drug concentration causing a 50% inhibition of the enzyme activity (IC_{50}) for each macrolide, expressed in μM and in mg/liter.

liter for azithromycin). At lower concentrations, no significant effect was seen with erythromycin A (apart from a transient increase at Day 1). Erythromycylamine caused a significant but barely time-dependent effect at 50 mg/liter but no effect at 10 mg/liter. Azithromycin caused a time-dependent effect at 10 mg/liter, but almost no effect at 2 mg/liter. The phospholipidosis was further characterized by analyzing the respective contribution of the main individual phospholipids. Figure 7 shows the results obtained from cells incubated 3 days with azithromycin at 50 mg/liter. A significant rise was observed for all phospholipids examined, except sphingomyelin and lysophosphatidylcholine. Yet, the importance of the rise varied markedly among phospholipids, and, whereas the content in phosphatidylcholine was clearly the most increased in absolute value, that of phosphatidylinositol was the most important when expressed as percent of controls. Similar findings were made when analyzing the phospholipid composition of cells incubated with 250 mg/liter of erythromycylamine, erythromycin A, or roxithromycin (but see above for comment about the viability of these cells when exposed to this high concentration of roxithromycin).

DISCUSSION

The present data show that macrolide antibiotics have the potential to interact with negatively charged phospholipid bi-

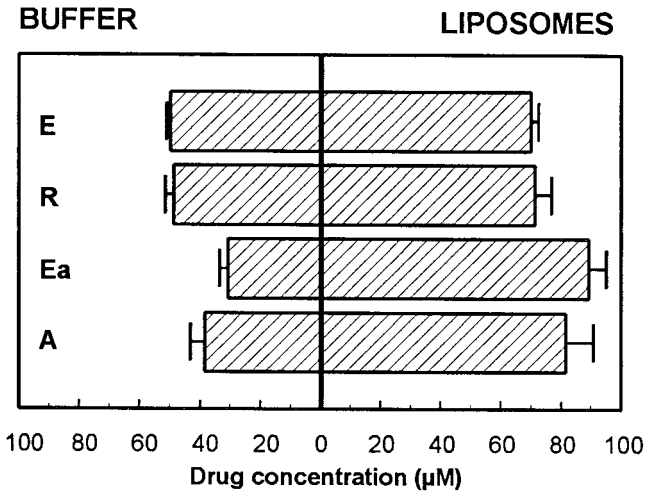

FIG. 5. Equilibrium dialysis of macrolides against negatively charged liposomes. Drugs (initial concentration, 120 μM) were placed on one side of the dialysis membrane (left chamber) and liposomes (concentration in total lipids, 15.7 mM; same composition as in Fig. 4) on the other side (right chamber). The diagram pictures the drug content of the two chambers after reaching equilibrium (buffer, liposome-free chamber; liposomes, liposome-containing chamber). The abcissa shows the drug concentration actually *measured* in the left chamber (plotted leftward), and the *calculated* drug concentration in the right chamber (plotted rightward) (see Materials and Methods]. Results are the means ± SD of five independent experiments. Statistical analysis: the Scheffe test disclosed a significant difference between erythromycylamine and azithromycin ($p < 0.01$) and between these two drugs and erythromycin and roxithromycin ($p < 0.001$).

layers, to bind to liposomes, and to inhibit the activities of lysosomal phospholipases in acellular systems. Moreover, our data confirm and extend previous data obtained with azithromycin on the ability of macrolides to cause phospholipidosis in cultured fibroblasts. This study thus provides a basis for delineating structure–effect relationships in this context, stressing the importance not only of a dicationic character but also of the specific localization of these charges when the drugs interact with a negatively charged monolayer.

At first glance, the position and orientation adopted by macrolides in a phosphatidylinositol monolayer (as well as that of the isolated molecules with respect to the hydrophobic–hydrophilic interface) may seem unlikely because it means that the *N*-3′ aminogroup is placed in the hydrophobic domain, an apparently surprising position. Generally speaking, the results of a conformational analysis need to be carefully compared with independent experimental data for validation. In the case of azithromycin, Montenez *et al.* (1996) demonstrated, by ^{31}P nuclear magnetic resonance spectroscopy, that the drug interacts with the phosphate heads of phospholipids, and, by fluorescence polarization studies, that it effectively penetrates in a negatively charged bilayer spanning across the hydrophobic–hydrophilic interface. Beyond these considerations, the present study actually strongly suggests that the position of a macrolide at a hydrophobic–hydrophilic interface is largely dictated by the properties of the macrocycle. This cycle has no frank hydrophobic and hydrophilic regions (e.g., in a phospholipid [Brasseur, *et al.*, 1984] or in a cationic amphiphile like *bis*-β-(diethylaminoethylether) hexestrol [Mingeot-Leclercq *et al.*, 1989]), which is denoted by the fact that the hydrophobic and hydrophilic centers of all molecules studied are very close to each other (<0.8Å). The position of the macrocycle at the interface is not easily modified, as demonstrated by the behavior of roxithromycin (which carries a methoxyethoxymethyl-

TABLE 2
Cellular Accumulation of Macrolides and Change in Total Phospholipid Content in Fibroblast Cells after 72 h Incubation

Drug	Extracellular concentration (mg/L)	Extracellular concentration (μM)	Drug accumulation: Cellular content (nmol/mg protein)	Drug accumulation: Apparent cellular-to-extracellular concentration ratio[a]	Change in total phospholipid content (% of control values)
Erythromycin A	10	13.6	0.84 ± 0.03	12.5 ± 0.5	95 ± 4
	50	80.5	3.98 ± 0.13	11.8 ± 0.4	97 ± 4
	250	402.5	n.d.	n.a.	156 ± 2[c]
Roxithromycin	10	11.9	1.60 ± 0.10	23.6 ± 1.4	95 ± 2
	50	60.0	7.60 ± 0.44	22.5 ± 1.3	97 ± 1
Erythromycylamine	10	13.6	1.53 ± 0.09	22.5 ± 1.3	101 ± 3
	50	68.0	7.43 ± 0.58	21.8 ± 1.7	121 ± 1[c]
	250	340.0	n.d.	n.a.	174 ± 3[c]
Azithromycin	10	13.3	5.37 ± 0.14	85.1 ± 2.2	137 ± 6[b]
	50	67.0	28.12 ± 0.66	89.2 ± 2.1	174 ± 6[c]

[a] See Materials and Methods.
[b] $p < 0.01$ (Student's *t* test).
[c] $p < 0.001$ (Student's *t* test).
n.d., not determined; n.a., not applicable.

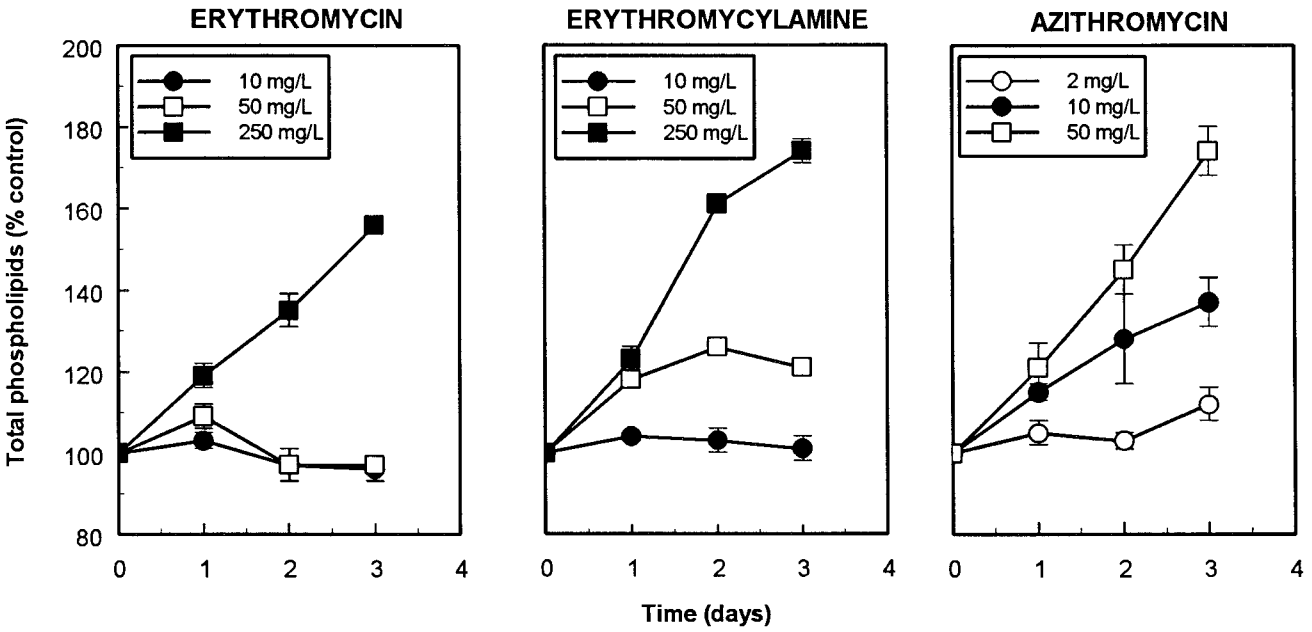

FIG. 6. Time evolution of the cellular content in total phospholipids of fibroblasts exposed to increasing concentrations of erythromycin, erythromycylamine, and azithromycin. The drug extracellular concentrations are given in each panel (note that the concentrations of erythromycin and erythromycylamine are five times larger than those of azithromycin). Results are expressed as percent of control values. Statistical analysis was as follows: for all three macrolides, a two-way ANOVA detected a significant effect of the time of incubation ($p < 0.001$), drug concentration ($p < 0.001$), and a combined effect of these two parameters ($p < 0.001$). Scheffe's test showed that a significant increase in total phospholipids was obtained for erythromycin from Day 1 at a concentration of 250 mg/liter ($p < 0.01$) for erythromycylamine from Day 1 at concentrations $\geq$ 50 mg/liter ($p < 0.001$) and for azithromycin from Day 2 at concentrations $\geq$ 10 mg/liter ($p < 0.01$ for 10 mg/liter and $p < 0.001$ for 50 mg/liter).

N-oxime side chain; it is however modified in dirithromycin, probably by the simultaneous presence of a secondary amine attracting this part of the molecule to the aqueous phase and of a methoxyethoxyethylidene side chain positioning it close to the hydrophobic zone). Finally, the transfer energies of the five molecules are very similar, whether protonated or not, indicating that the role played by the ionizable nitrogens is probably minor compared to that of other parts of the molecule, at least among the compounds studied. This quite surprising observation has been validated for azithromycin by simulation studies using virtual molecules with a demethylated desosamine. Those adopt a strikingly different conformation and orientation, with the protonated primary amine penetrating deeply in the hydrophilic zone. This suggests that the positioning of the *N*-3′ aminogroup of azithromycin in the hydrophobic domain should be largely due to the masking of its positive charge by the two methylgroups (Montenez *et al.*, 1996).

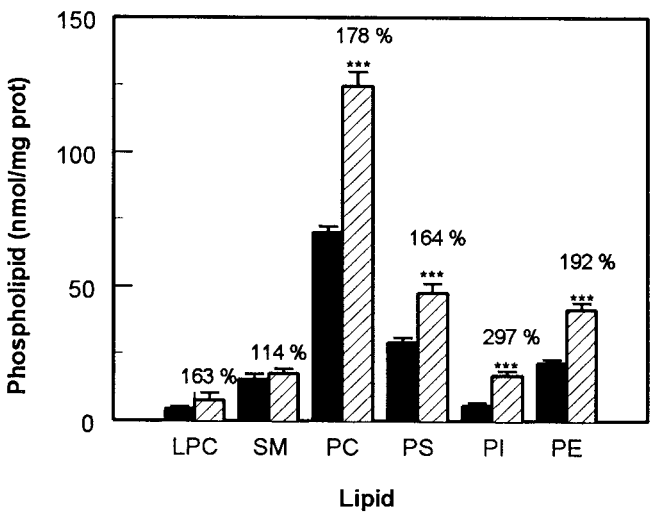

FIG. 7. Patterns of phospholipids (LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; and PE, phosphatidylethanolamine) in fibroblasts incubated during 72 h in the absence of drug (closed bars) or in the presence of 50 mg/liter azithromycin (hatched bars). The increase of phospholipid content is also given as percent of control value above each bar. ***$p < 0.001$ by the Student's *t* test in comparison with the matching control.

Since the overall positions of the molecules are similar, the difference in amounts of drug bound to bilayers and inhibitory potencies toward lysosomal phospholipase A_1 has to depend on the specific nature of the interactions each macrolide can establish with key parts of the phospholipids (we have not addressed here the possibility of a direct interaction of the drugs with the enzyme since this was largely ruled out by our previous studies showing no inhibition of phospholipase A_1 when assays are made on detergent-dispersed substrate [Van Bambeke *et al.*, 1996]; the possibility of forming ternary complexes drug–phospholipids–enzyme cannot however be dismissed). We suggested earlier that azithromycin inhibits phospholipase A_1 activity toward phosphatidylcholine embedded in

phosphatidylinositol-containing bilayers by neutralizing the negative surface charges necessary for optimal activity of the enzyme (Van Bambeke *et al.,* 1996). In this context, the difference in inhibitory potencies observed here between the mono- and dicationic macrolides appear related to their differential ability to position one of their aminogroup close enough from the phosphogroups of phosphatidylinositol. Thus, the conformational analysis and the binding studies predict that all macrolides should be inhibitory if tested at sufficiently large concentrations but that those that can position an aminogroup close to a phosphogroup should be much more potent inhibitors. Properties of the aminated functions other than their simple location or number could, however, play a role in this respect. For example, the strength of the basic function may also affect the interaction with phospholipids. We must also take into account the fact that the apparent pK_as of the aminogroups in interaction with the lipids may be very different from those of the drug in solution (Chung *et al.,* 1985) (determination of this pK_a could be checked by combination of the bell-shaped D-pH diagram with the Henderson-Hasselbach diagram [see Krämer and Wunderli-Allenspach, 1996 for methodology and details] or by ^2H NMR spectroscopy). But, whatever the exact basicity of amines, it remains that all the macrolides currently used in clinics possess the same aminated group on their desosamine, making structure–activity relationships studies unrealizable. At this stage, we can therefore only compare the properties of the amines substituting C-9 in erythromycylamine and in position 9a of azithromycin, respectively. The fact that no major differences were observed in the inhibitory potential of these molecules toward phospholipase A_1 suggests that the presence of a positive charge near the phosphate heads rather than the exact nature of this cation is critical in this context. A maximal inhibition is indeed obtained for all four drugs for a drug:phosphatidylinositol ratio of $\approx$0.2 for the monocationic macrolides and of only $\approx$0.1 for the dicationic macrolides, suggesting that a charge per charge neutralization is the important factor for inhibition. This point should be, however, further investigated since we do not know how much phosphatidylinositol is accessible in our system to both the macrolide and to the phospholipase A_1 so that an absolute stoichiometric ratio cannot be calculated. The lack of labeled compound has unfortunately prevented us from performing detailed kinetic studies of the comparative binding of macrolides to liposomes, but an interesting development, achievable with the available material, would be a direct measure of the reduction of the surface potential of the vesicles in the presence of the antibiotics.

If the *in vitro* data are now used to appraise the situation prevailing in the cultured cells, it becomes clear that the extent of the phospholipidosis observed with the different macrolides is related both to their capacity to accumulate in cells (toxicokinetic parameter) and to their intrinsic inhibitory potency toward lysosomal phospholipases (toxicodynamic parameter). The lysosomal nature of the phospholipidosis described here has been demonstrated for azithromycin (Van Bambeke *et al.,* 1996) and it may reasonably be assumed that this also holds true for the other macrolides. Phospholipase A_1 plays the major role in the lysosomal breakdown of phosphatidylcholine, the main structural phospholipid that is indeed found in the largest excess. The preferential increase in phosphatidylinositol content on a relative basis may indicate a faster turnover of this phospholipid, or a differential effect on its specific catabolic pathway, two issues that may be experimentally addressed. Quantitative estimations are in agreement with these conclusions. Since the largest part of macrolides accumulated in cells is in the lysosomes (Carlier *et al.,* 1994), it is clear that the drug concentrations reached within these organelles are sufficient to cause enzyme inhibition. Interestingly enough, the excess of phosphatidylinositol and phosphatidylserine found in cells matches almost mole for mole the amounts of macrolide stored, which would reinforce our conclusion that negatively charged phospholipids probably constitute a key target for macrolides. The comparison of erythromycylamine with azithromycin is instructive in this respect since it demonstrates that the less marked phospholipidosis obtained in cells with this antibiotic, compared to azithromycin, essentially results from its specific toxicokinetic properties (lower accumulation). The present study offers no simple explanation for this difference (see Carlier *et al.,* 1987; de Duve *et al.,* 1974 for a model and a discussion of the accumulation process of weak organic bases in cells and lysosomes), which nevertheless points to potentially interesting developments, since it predicts that lowering macrolide accumulation may be one way to reduce its potential toxicity. In contrast, the differences in phospholipidosis caused by roxithromycin and erythromycylamine appear more related to their toxicodynamic characteristics (intrinsic ability to inhibit phospholipase activity). Putting all these observations together leads us to the conclusion that the propensity of a given macrolide to cause phospholipidosis must be understood as a conjunction of its ability to accumulate into the cells and to inhibit phospholipase activity.

Moving from the mere discussion of the data, we suggest this study may open a series of potentially useful perspectives. First, it reinforces our earlier proposals that lysosomal breakdown of phospholipids could be regulated by the amount of negatively charged phospholipids present in the membranes segregated within these organelles. While drugs would appear as exogenous modulators, endogenous modulators might also exist. Second, the approaches used here could be used and developed to better prospectively assess the potential side effects of existing and future macrolides, including in some of their specific uses beyond those envisaged by conventional toxicological approaches (Van Bambeke *et al.,* 1998, for instance, demonstrated that prolonged incubation of fibroblasts at extracellular concentrations of azithromycin similar to those expected to be observed in AIDS patients treated for *Mycobacterium avium intracellulare* infection caused an easily detectable phospholipidosis). Third, our study, together with that of Montenez *et al.* (1996), also suggests that metabolites appearing *in vivo* (and especially the *N*-demethylated derivatives,

which arise through hepatic biotransformation of most macrolides; see, e.g., Puri and Lassman, 1987; Luke and Foulds, 1997) could be worthwhile to investigate since their interaction with phospholipids could be largely different from those of the present drug because of the unshielding of the *N*-3′ aminogroup. Fourth, more profound modifications of the drug, like the removal of the cladinose, as is also observed in the biotransformation of macrolides or is purposely made with the new family of ketolides (Agouridas *et al.*, 1997), could result in very different types of interactions. These may be of critical importance because drugs developed in this direction are characterized by a still larger level of cellular accumulation than is observed with azithromycin.

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REFERENCES

Agouridas, C., Bonnefoy, A., and Chantot, J. F. (1997). Antibacterial activity of RU64004 (HMR 3004), a novel ketolide derivative active against respiratory pathogens. *Antimicrob. Agents Chemother.* **41,** 2149–2158.

Aubert-Tulkens, G., Van Hoof, F., and Tulkens, P. (1979). Gentamicin-induced lysosomal phospholipidosis in cultured rat fibroblasts. *Lab. Invest.* **40,** 481–491.

Bartlett, G. R. (1959). Phosphorus assay in column chromatography. *J. Biol. Chem.* **234,** 466–468.

Brasseur, R. (1990). TAMMO: Theoretical analysis of membrane molecular organization. In *Molecular Description of Biological Membrane Components by Computer-Aided Conformational Analysis,* Vol. I/1.A.6. (R. Brasseur, Ed.), pp. 203–219. CRC Press, Boca Raton, FL.

Brasseur, R., Deleers, M., Malaisse, W. J., and Ruysschaert, J. M. (1982). Conformational analysis of the calcium-A23187 complex at a lipid-water interface. *Proc. Natl Acad. Sci. USA* **79,** 2895–2897.

Brasseur, R., Deleers, M., and Ruysschaert, J. M. (1986). Mode of organization of amphiphilic molecules at lipid–water interface: A conformational analysis. *J. Colloid. Interface Sci.* **114,** 277–281.

Brasseur, R., Goormaghtigh, E., and Ruysschaert, J. M. (1981). Theoretical conformational analysis of phospholipid bilayers. *Biochem. Biophys. Res. Commun.* **103,** 301–310.

Brasseur, R., Laurent, G., Ruysschaert, J. M., and Tulkens, P. M. (1984). Interactions of aminoglycoside antibiotics with negatively charged lipid layers: Biochemical and conformational studies. *Biochem. Pharmacol.* **33,** 629–637.

Brasseur, R., Vandenbranden, M., Cornet, B., Burny, A., and Ruysschaert, J. M. (1990). Orientation into the lipid bilayer of an asymmetric amphipathic helical peptide located at the *N*-terminus of viral fusion proteins. *Biochim. Biophys. Acta* **1029,** 267–273.

Bright, G. M., Nagel, A. A., Bordner, J., Desa, K. A., Dibrino, J. N., Nowakowska, J., and Sciavolino, F. C. (1988). Synthesis, *in vitro* and *in vivo* activity of novel 9-deoxo-9a-AZA-9a-homoerythromycin A derivatives: A new class of macrolide antibiotics, the azalides. *J. Antibiot.* **41,** 1029–1047.

Carlier, M. B., Garcia-Luque, I., Montenez, J-P., Tulkens, P. M., and Piret, J. (1994). Accumulation, release and subcellular localization of azithromycin in phagocytic and non-phagocytic cells in culture. *Int. J. Tissue React.* **16,** 211–220.

Carlier, M. B., Zenebergh, A., and Tulkens, P. M. (1987). Cellular uptake and subcellular distribution of roxithromycin and erythromycin in phagocytic cells. *J. Antimicrob. Chemother.* **20(B),** 47–56.

Chantot, J.-F., Bryskier, A., and Gasc, J.-C. (1986). Antibacterial activity of roxithromycin: A laboratory evaluation. *J. Antibiot.* **39,** 660–668.

Chung, L., Kaloyanides, G., McDaniel, R., McLaughin, A., and McLaughin, S. (1985). Interaction of gentamicin and spermine with bilayer membranes containing negatively-charged phospholipids. *Biochemistry* **24,** 442–452.

Counter, F. T., Ensminger, P. W., Preston, D. A., Wu, C. Y., Greene, J. M., Felty-Duckworth, A. M., and Kirst, H. A. (1991). Synthesis and antimicrobial evaluation of dirithromycin (AS-E-136; LY237216), a new macrolide antibiotic derived from erythromycin. *Antimicrob. Agents Chemother.* **35,** 1116–1126.

de Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P., and Van Hoof, F. (1974). Lysosomotropic agents. *Biochem. Pharmacol.* **23,** 2495–2531.

Djokic, S., Kobrehel, G., Lopotar, N., Kamenar, B., Nagl, A., and Mrvos, D. (1988). Erythromycin series. Part 13. Synthesis and structure elucidation of 10-dihydro-10-deoxo-11-methyl-11-azerythromycin A. *J. Chem. Res.* (Summary) 152; (Manuscript) 1239.

Foulds, G., Shepard, R. M., and Johnson, R. B. (1990). The pharmacokinetics of azithromycin in human serum and tissues. *J. Antimicrob. Chemother.* **25(A),** 73–82.

Grove, D. C., and Randall, W. A. (Eds.) (1955). *Assay Methods of Antibiotics.* Medical Encyclopedia, New York.

Harris, D. R., Mc Geachin, S. G., and Mills, H. H. (1965). The structure and stereochemistry of erythromycin A. *Tetrahedron Lett.* **11,** 679–685.

Kirst, H. A., Creemer, L. C., Paschal, J. W., Preston, D. A., Alborn, W. E., Counter, F. T., and Greene, J. M. (1995). Antimicrobial characterization and interrelationships of dirithromycin and epidirithromycin. *Antimicrob. Agents Chemother.* **39,** 1436–1441.

Kirst, H. A., Wind, J. A., Leeds, J. P., Willard, K. E., Debono, M., Bonjouklian, R., and Counter, F. T. (1990). Synthesis and structure–activity relationships of new 9-*N*-alkyl derivatives of 9(*S*)-erythromycylamine. *J. Med. Chem.* **33,** 3086–3094.

Kodavanti, U. P., and Mehendale, H. M. (1990). Cationic amphiphilic drugs and phospholipid storage disorder. *Pharmacol. Rev.* **42,** 327–354.

Kotretsou, S., Mingeot-Leclercq, M.-P., Constantinou-Kokotou, V., Brasseur, R., Georgiadis, M. P., and Tulkens, P. M. (1995). Synthesis and antimicrobial and toxicological studies of amino acid and peptide derivatives of kanamycin A and netilmicin. *J. Med. Chem.* **38,** 4710–4719.

Krämer, S. D., and Wunderli-Allenspach, H. (1996). The pH-dependence in the partitioning behaviour of (RS)-[^{3}H]propanol between MDCK cell lipid vesicles and buffer. *Pharm. Res.* **13,** 1851–1855.

Laurent, G., Carlier, M. B., Rollman, B., Van Hoof, F., and Tulkens, P. (1982). Mechanism of aminoglycoside-induced lysosomal phospholipidosis: *In vitro* and *in vivo* studies with gentamicin and amikacin. *Biochem. Pharmacol.* **31,** 3861–3870.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Luger, P., and Maier, R. (1979). Molecular structure of 9-deoxy-11-deoxy-9–11-(imino(2-(2-methoxyethoxy)ethylidene)oxy)-(9*S*)-erythromycin, a new erythromycin derivative. *J. Cryst. Mol. Struct.* **9,** 329–338.

Luke, D. R., and Foulds, G. (1997). Disposition of oral azithromycin in humans. *Clin. Pharmacol. Ther.* **61,** 641–648.

Massey, E. H., Kitchell, B., Martin, L. D., Gerzon, K., and Murphy, H. W. (1970). Erythromycylamine. *Tetrahedron Lett.* **2,** 157–160.

Mingeot-Leclercq, M. P., Laurent, G., and Tulkens, P. M. (1988). Biochemical mechanism of aminoglycoside-induced inhibition of phosphatidylcholine hydrolysis by lysosomal phospholipases. *Biochem. Pharmacol.* **37,** 591–599.

Mingeot-Leclercq, M. P., Schanck, A., Ronveaux-Dupal, M. F., Deleers, M., Brasseur, R., Ruysschaert, J. M., and Tulkens, P. M. (1989). Ultrastructural, physico-chemical and conformational study of the interactions of gentamicin and *bis*(beta-diethylaminoethylether)hexestrol with negatively charged phospholipid bilayers. *Biochem. Pharmacol.* **38,** 729–741.

Mingeot-Leclercq, M. P., Van Schepdael, A., Brasseur, R., Busson, R., Vanderhaeghe, H. J., Claes, P. J., and Tulkens, P. M. (1991). New derivatives of Kanamycin B obtained by modifications and substitutions in position 6‘‘. II. *In vitro* and computer-aided toxicological evaluation, with respect to interactions with phosphatidylinositol. *J. Med. Chem.* **34,** 1476–1482.

Montenez, J. P., Van Bambeke, F., Piret, J., Schanck, A., Tulkens, P. M., Brasseur, R., and Mingeot-Leclercq, M. P. (1996). Interaction of the macrolide azithromycin with phospholipids. II. Biophysical and computer-aided conformational studies. *Eur. J. Pharmacol.* **314,** 215–227.

Puri, S. K., and Lassman, H. B. (1987). Roxithromycin: A pharmacokinetic review of a macrolide. *J. Antimicrob. Chemother.* **20(B),** 89.

Sheldrick, G. M., Kojic-Prodic, B., Banic, Z., Kobrehel, G., and Kujundzic, N. (1995). Structure of 9-deoxo-9a-*N*-[*N*′-(4-pyridyl)-carbamoyl]-9a-aza-9a-homoerythromycin A and conformational analysis of analogous 9a-aza 15-membered azalides in solide state. *Acta Crystallogr., Sect. B* **B51,** 358–366.

Tulkens, P. M. (1991). Intracellular distribution and activity of antibiotics. *Eur. J. Clin. Microbiol. Infect. Dis.* **10,** 100–106.

Tulkens, P. M., Beaufay, H., and Trouet, A. (1974). Analytical fractionation of homogenates from cultured rat embryo fibroblasts. *J. Cell Biol.* **63,** 383–401.

Tulkens, P. M., and Trouet, A. (1978). The uptake and intracellular accumulation of aminoglycoside antibiotics in lysosomes of cultured rat fibroblasts. *Biochem. Pharmacol.* **27,** 415–424.

Van Bambeke, F., Gerbaux, C., Michot, J.-M., Bouvier d’Yvoire, M., Montenez, J. P., and Tulkens, P. M. (1998). Lysosomal alterations induced in cultured rat fibroblasts by long-term exposure to low concentrations of azithromycin. *J. Antimicrob. Chemother.* **42,** 761–767.

Van Bambeke, F., Montenez, J. P., Piret, J., Tulkens, P. M., Courtoy, P. J., and Mingeot-Leclercq, M. P. (1996). Interaction of the macrolide azithromycin with phospholipids. I. Inhibition of lysosomal phospholipase A_1 activity. *Eur. J. Pharmacol.* **314,** 203–214.

Vassault, A. (1987). Lactate dehydrogenase. In *Methods of Enzymatic Analysis, Volume III/Enzyme I Oxydoreductases, Transferases* (Bergmeyer H. U., Ed.), pp. 118–126. VHC Publishers, Weinheim, RFA.

Wiley, P., Gerzon, K., Flynn, E. H., Sigal, M. V., Jr, Weaver, O., Quarck, U. C., and Monahan, R. (1957). Erythromycin. X. Structure of erythromycin. *J. Am. Chem. Soc.* **79,** 6062–6069.