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Interaction of the macrolide azithromycin with phospholipids. I. Inhibition of lysosomal phospholipase A_1 activity

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Abstract

Azithromycin, the first clinically developed dicationic macrolide antibiotic, displays an exceptional accumulation in lysosomes of cultured cells. In fibroblasts incubated with 50 mg/l (66.6 μM), it induces a distinct lysosomal phospholipidosis as evidenced by biochemical and ultrastructural criteria, which strikingly resembles alterations described previously with gentamicin, a pentacationic aminoglycoside antibiotic which inhibits the lysosomal catabolism of phospholipids. We show that both drugs inhibit, in an equimolar manner, the activity of phospholipase A_1 (assayed for phosphatidylcholine, included in negatively charged liposomes), in a way consistent with the model of 'charge neutralization' proposed already for gentamicin (Mingeot-Leclercq et al., 1988, Biochem. Pharmacol. 37, 591). Both drugs bind to negatively charged liposomes. Yet, in spite of this binding, azithromycin does not induce aggregation or fusion of negatively charged vesicles, under conditions in which gentamicin (or spermine, a fully hydrophilic polycation) causes a massive aggregation, and bis(β-diethylaminoethylether)hexestrol (a dicationic amphiphile) causes fusion. The molecular interactions of azithromycin with acidic phospholipids are further examined in a companion paper.

Keywords: Azithromycin; Macrolide; Gentamicin; Aaminoglycoside; Lysosomal phospholipase; Phospholipidosis; Membrane aggregation

1. Introduction

Azithromycin (Fig. 1) is the first clinically developed antibiotic in a new subclass of the macrolides, the azalides, characterized by the expansion of the 14-membered aglycone ring of erythromycin with an endocyclic ionizable nitrogen (Bright et al., 1988; Djokic et al., 1988). Compared with erythromycin, azithromycin displays several interesting properties, among which an exceptionally high level of accumulation and retention in cells and tissues (Girard et al., 1987; Gladue et al., 1989; Foulds et al., 1990). Cell fractionation studies reveal that most of the azithromycin accumulated by cells and tissues is largely stored in lysosomes (Gladue and Snider, 1990; Shepard et al., 1992; Carlier et al., 1994), such as that found for other macrolides (Carlier et al., 1987; Villa et al., 1988). Analysis of the cell uptake kinetics of azithromycin, and theoretical considerations on the cellular disposition of weak organic bases (De Duve et al., 1974) suggest that its huge lysosomal accumulation results from the proton-driven segregation of the dicationic form of the molecule within the acidic milieu of these organelles (≈ 5; Ohkuma and Poole, 1978). The drug stored in lysosomes remains nevertheless potentially active, and largely bioavailable, inside as well as outside of lysosomes in view of its effectiveness against most extracellular and intracellular pathogens within its spectrum of antibacterial activity (see Girard et al., 1987, for early animal data and Steigbigel, 1995, for a recent clinically oriented overview of azithromycin properties). Yet, questions may be raised concerning the potential development of modifications of lysosome properties associated with these high levels of drug. It is indeed intriguing that the efflux of azithromycin from lysosomes and cells is considerably slower than that of most other macrolides (Gladue et al., 1989; Kirst and Sides, 1989b). Shepard et al. (1992) reported that the administration of

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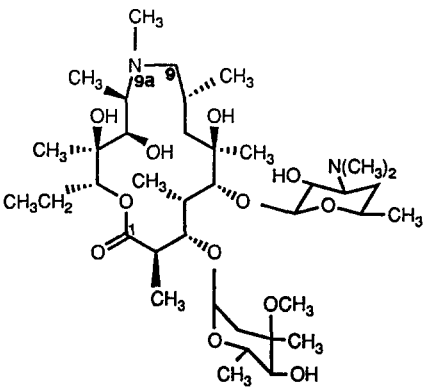

Fig. 1. Structure of azithromycin. Compared to erythromycin A, azithromycin is characterized by the removal of the oxo radical from C_9 and by ring expansion with a methylated endocyclic nitrogen (position 9a). The commonly accepted systematic name of azithromycin, showing that it derives from erythromycin, is 9-deoxo-9a-methyl-9a-azahomoerythromycin A (atoms are numbered counterclockwise from the endocyclic carbonyl; this nomenclature eases the structural comparisons with erythromycin and other antibiotics in the family of the 14-membered ring macrolides (Bright et al., 1988; Kirst and Sides, 1989a)). Another nomenclature, giving position 1 to the lactonic O and insisting on the ring expansion, is 10-dihydro-10-deoxo-11-methyl-11-azaerythromycin A (Djokic et al., 1988). See also the companion paper (Montenez et al., 1996) for a systematic chemical name of azithromycin that strictly follows the IUPAC rules.

large doses of azithromycin causes the appearance, in rats and dogs, of polylamellar bodies in several tissues and a decrease in the buoyant density of liver lysosomes. The latter change was also observed by Carlier et al. (1994) in fibroblasts cultured with azithromycin.

The present paper documents that azithromycin causes a phospholipidosis in cultured fibroblasts associated with obvious changes in the ultrastructural appearance of lysosomes. It also establishes that azithromycin interacts in vitro at acid pH with phospholipid bilayers in a way that may account for the development of this phospholipidosis. The analysis is based on a systematic comparison with gentamicin, a polycationic aminoglycoside antibiotic of entirely different chemical structure, but which also accumulates in lysosomes of cultured fibroblasts (Tulkens and Trouet, 1978) and induces a typical lysosomal phospholipidosis (Aubert-Tulkens et al., 1979). Additional comparison is made with spermine, a hydrophilic polycation and with bis(β-diethylaminoethylether)hexestrol (DEH), a dicationic amphiphile originally selected as a anticholesterolemic drug. All these compounds were shown to inhibit lysosomal phospholipases in vitro by interacting with negatively charged phospholipids in membranes (see Mingeot-Leclercq et al., 1995, for review). The companion paper (Montenez et al., 1996) complements these in vitro studies of the azithromycin-phospholipid interactions. It further describes biophysical approaches and presents a computer-aided conformational analysis of mixed monolayers of azithromycin and phosphatidylinositol.

2. Materials and methods

2.1. Cell culture experiments

Fibroblasts were obtained from trypsinized rat embryo carcasses and used at the second or third subculture (Tulkens et al., 1974). For biochemical assays, cells were washed twice, collected by scraping, and pelleted by centrifugation. Lipids were extracted according to Bligh and Dyer (1959) and total lipid phosphorus measured on the chloroform phase by the method of Bartlett (1959). Protein was measured by the method of Lowry et al. (1951) with serum albumin as a standard. Electron microscopy was performed on cells fixed in situ and processed as described by Montenez et al. (1994).

2.2. Liposome experiments

2.2.1. Liposome preparation

Two types of liposomes were used, viz. small unilamellar vesicles (SUV), prepared by sonication (Laurent et al., 1982), and large unilamellar vesicles (LUV), prepared by extrusion (Van Bambeke et al., 1993). Small unilamellar vesicles displayed a mean diameter of ≈ 25 nm [95% range: 15 and 90 nm, as determined by electron microscopic examination of replicas of freeze-fractured preparations (Mingeot-Leclercq et al., 1989)]. They were used for drug binding and phospholipase A_1 inhibition experiments so that the results of these studies are directly comparable with those of previous studies with gentamicin and bis(β-diethylaminoethylether)hexestrol (DEH) (Mingeot-Leclercq et al., 1988, 1990). Large unilamellar vesicles displayed a larger mean diameter (≈ 100 nm) and a more homogenous size (see Section 3). Showing less internal tension than small unilamellar vesicles, large unilamellar vesicles are, therefore, more suited to fusion studies (Hope et al., 1985), and were accordingly used for fluorescence dequenching experiments and light scattering spectroscopy, two techniques which test for fusion and/or aggregation of vesicles.

Routinely, all liposomes were made of cholesterol, phosphatidylcholine, sphingomyelin, and phosphatidylinositol. Preparations consistently contained 1 mol of cholesterol for 2 mol of phospholipids. Unless stated otherwise, phosphatidylcholine was added at a ratio of 4 mol per 7 mol of the two other phospholipids combined (11 mol total phospholipids). The negative charge of the liposomes was varied by increasing the phosphatidylinositol content from 1 to 6 mol per 11 mol of total phospholipids, while at the same time the sphingomyelin content was decreased from 6 to 1 mol in order to keep the phospholipid : cholesterol ratio constant. To enhance the fusogenicity of the vesicles, phosphatidylethanolamine was added in partial replacement of sphingomyelin (up to 2.3 mol per 11 mol of total phospholipids, reducing thereby the maximal content of the vesicles in sphingomyelin to 1.7) and by partial re-

placement of phosphatidylcholine thereafter (up to a maximum of 4 mol of phosphatidylethanolamine per 11 mol of total phospholipids, reducing thereby the phosphatidylcholine content to a minimum of 2.3 mol per 11 mol of phospholipids). For these vesicles, phosphatidylinositol was added at a constant ratio of 3:11 with respect to the sum of all the phospholipids.

The required quantities of lipids were dissolved in chloroform/methanol (2:1, v/v) in a round-bottomed flask. The solvent was evaporated under vacuum (Rotavapor Buchi RE-111; Buchi, Flawil, Switzerland) to obtain a thin film of lipids which was dried overnight in a vacuum dessicator. Lipids were then suspended in the required volume of buffer (generally, 40 mM acetate buffer, pH 5.4, except for the studies examining the effect of the ionic strength on drug binding), and incubated in a nitrogen atmosphere at 37°C during 1 h.

Small unilamellar vesicles were obtained by sonication at 4°C under a stream of nitrogen with a Branson Sonifier (Branson Sonic Power, Danbury, CT, USA) set at 50 W for 5×2 min with 1-min cooling intervals, or until the opaque suspension became translucent. The preparations were then centrifuged at $850 \times g$ for 15 min to remove particulate matter. To obtain large unilamellar vesicles, the lipid suspension was submitted to 5 cycles of freeze-thawing and thereafter extruded 10 times in a Thermobarrel Extruder (Lipex Biomembranes, Vancouver, Canada) under a nitrogen pressure of 17 bars through 2 polycarbonate filters with a pore size of 100 nm (Nucleopore; Costar Europe, Badhoevedorp, The Netherlands). The size of the vesicles was routinely checked as described below. The phospholipid content of each final preparation was determined by phosphorus assay (Bartlett, 1959) and the concentration of liposomes adjusted accordingly for each type of experiment. Liposomes were stored under nitrogen at 4°C and used within a week.

2.2.2. Binding studies

Binding of azithromycin and gentamicin to small unilamellar vesicles was investigated by equilibrium dialysis using a Dianorm apparatus (Dianorm Geräte, München, Germany), consisting of sets of 200 μl twin cells made of Teflon and separated by a Diachema flat dialysis membrane (M_r cut-off: 5000) as described earlier (Mingeot-Leclercq et al., 1990). The drug was introduced in the left chamber at a 120 μM concentration, and small unilamellar vesicles in the right chamber at a total lipid concentration of 10 g/l (15.7 mM). Dialysis was performed for 5 h at 37°C under constant rotation (control experiments showed that equilibrium dialysis was achieved after 3 h). Drugs were assayed in the left chamber (containing no liposomes) before dialysis ($D_{initial}$) and after dialysis ($D_{final} = D_{free}$) by reaction with fluorescamine for gentamicin (Udenfriend et al., 1972), or by a disc-plate microbiological assay using *Bacillus subtilis* (Grove and Randall, 1955) for azithromycin. The apparent concentration of the total drug in the right chamber (free + bound [D_{total}]) was calculated as $D_{initial} - D_{free}$. In the absence of liposomes, D_{free} had a value equal to 0.5 ± 0.02 of $D_{initial}$.

2.2.3. Determination of lysosomal phospholipase A_1 activity

Activity of phospholipase A_1 (phosphatidate-1-acylhydrolase, EC 3.1.1.32) was measured using as enzyme source a soluble fraction of purified liver lysosomes isolated from rats treated with Triton WR 1339 as previously described by Trouet (1974). We followed the release of [^{14}C] β-lysophosphatidylcholine from 1-palmitoyl, 2-[1-^{14}C]oleoyl phosphatidylcholine (140 mCi/mol of phosphatidylcholine) included in small unilamellar vesicles (final concentration: 8 mM in total lipids) as described by Carlier et al. (1983), except that the final concentration of the buffer (used for preincubating the drugs with the liposomes and for the subsequent enzymatic assays) was set at 40 mM as described by Mingeot-Leclercq et al. (1988). All measurements were made in triplicate.

2.2.4. Determination of the aggregating and fusogenic potencies by octadecylrhodamine B chloride (R_{18}) fluorescence dequenching

These studies were made exactly, as described earlier (Van Bambeke et al., 1995), using a mixture of labeled and unlabeled large unilamellar vesicles (final concentration in lipids: 5 μM), and recording the increase in fluorescence due to the dequenching of R_{18} upon its dilution (Hoekstra et al., 1984). We followed the *rate* of the fluorescence dequenching to distinguish the fusion of the liposomes (which causes a rapid increase in the fluorescence signal) from their aggregation (which causes a slow increase), based on the effects observed previously with melittin and spermine, respectively. Labeled and unlabeled liposomes were mixed at a ratio of 1:4 and the fluorescence of the preparation was followed at room temperature during 30 min, on a Perkin-Elmer LS30 fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, UK) using an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

2.2.5. Determination of the size of liposomes

The apparent mean diameter of large unilamellar vesicles was determined by quasielastic light scattering spectroscopy (Mazer et al., 1979) using a Coulter Nano Sizer N_4MD (Coulter Electronics, Luton, UK) as described earlier (Mingeot-Leclercq et al., 1990). The liposome concentration was set at 65 μM (total lipids). Fluctuation of light scattering was measured at an angle of 90°, with monodisperse latex particles of 100 and 800 nm diameter as control. Data were analyzed in the 'unimodal mode', i.e., yielding a single, apparent mean diameter for all particles present in the population. Size dispersion was estimated by recording the standard deviation provided by the instrument, or in case of polydisperse populations, by recording the 95% size limit interval.

2.3. Materials

Azithromycin, supplied as the dihydrate free base by Pfizer (Central Research, Groton, CT, USA), was dissolved in 0.1 M HCl and brought to pH 5.4 at a final concentration of 50 g/l (66.5 mM). Gentamicin (a mixture of components C_1, C_{1a} and C_2/C_{2a} in a molar ratio of $\approx$ 27:21:52) was supplied as sulfate salt for research purposes by Schering Plough (Kenilworth, NJ, USA). All concentrations of azithromycin and gentamicin refer to the corresponding free base. The other specific products were obtained as follows: egg yolk phosphatidylcholine, egg yolk phosphatidylethanolamine, and wheat germ phosphatidylinositol (grade 1 products) from Lipid Products (Nr Redhill, UK); bovine brain sphingomyelin and cholesterol from Sigma (St. Louis, MO, USA); radiolabeled phosphatidylcholine (1-palmitoyl-2 [1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine; 52 mCi/mmol) from Amersham International (Amersham, UK); spermine and melittin from Sigma; and octadecylrhodamine B chloride (R_{18}) from Molecular Probes (Eugene, OR, USA). Other reagents were obtained from E. Merck (Darmstadt, Germany) and were of analytical grade. Pregnant rats for fibroblast cultures were of the Wistar strain and obtained at the Animalerie facultaire of the Université Catholique de Louvain (Brussels, Belgium).

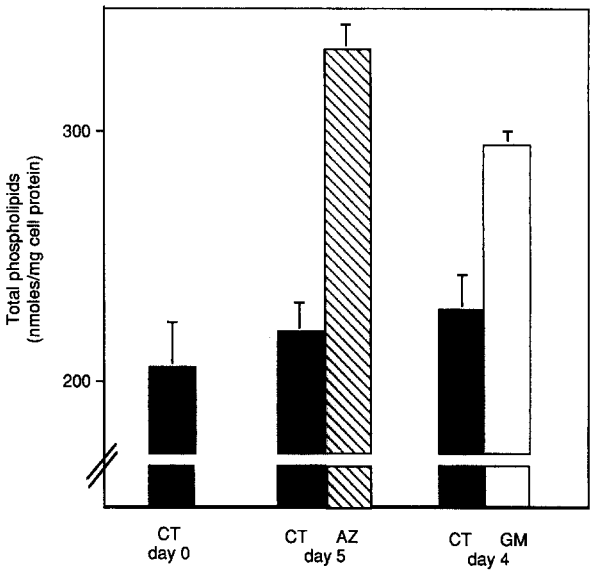

Fig. 3. Influence of azithromycin (hatched bars) and gentamicin (open bars) on the content of cultured fibroblasts in total lipid phosphorus (control = closed bars). Cells were incubated for 3 days with azithromycin (50 mg/l; 66 μM) or for 4 days with gentamicin (500 mg/l; 1.1 mM). Values are given as mean $\pm$ S.D. ($n = 8$ for control at day 0; $n = 3$ for all other series of data points).

3. Results

3.1. Cell culture studies

Fig. 2 shows the typical morphological appearance of fibroblasts after incubation with 13.3 μM azithromycin (10 mg/l) for 24 h. Most conspicuously, lysosomes were more abundant, looked grossly enlarged, and were partially filled by a mixture of concentric, osmiophilic inclusions, which upon higher magnification were clearly of lamellar structure (pseudomyelinic figures or 'myeloid bodies'). The remaining part of the lysosomal matrix was made of a mixture of less electron-dense heterogenous material and large electron-lucent zones. Other cell structures looked

Fig. 2. Ultrastructure of lysosomes from rat embryo fibroblasts cultivated for 24 h in the presence of azithromycin (10 mg/l; 13.2 μM). The picture illustrates the typical accumulation of lysosomes filled with concentric, lamellar inclusions (pseudomyelinic figures, also called 'myeloid bodies'). The lamellar aspect of these structures is clearly visible upon examination at high magnification (inset). Magnifications: 20000 $\times$; inset, 340000 $\times$. Bars: 1 μm and 50 nm, respectively.

essentially normal. This appearance of the cells prompted us to assay the cell homogenates for polar lipids.

As will be reported in detail elsewhere (Montenez, 1996; Montenez et al., manuscripts in preparation), azithromycin caused indeed a time- and dose-dependent increase in the content of the cells in lipid phosphorus. For the sake of comparison, we examined the influence of gentamicin, based on our earlier report that this drug also causes time- and dose-dependent phospholipidosis in fibroblasts. Fig. 3 shows typical data obtained with cells incubated with 66.6 μM azithromycin (50 mg/l) for 3 days and with 1.1 mM gentamicin (500 mg/l) for 4 days. These conditions allowed to obtain a grossly similar cellular drug content for the two antibiotics [22.2 $\pm$ 0.5 μg/mg and 6.2 $\pm$ 0.4 μg/mg of cell protein for azithromycin and gentamicin, respectively ($n = 3$)], and caused in both cases a comparable clear-cut phospholipid overload of the fibroblasts.

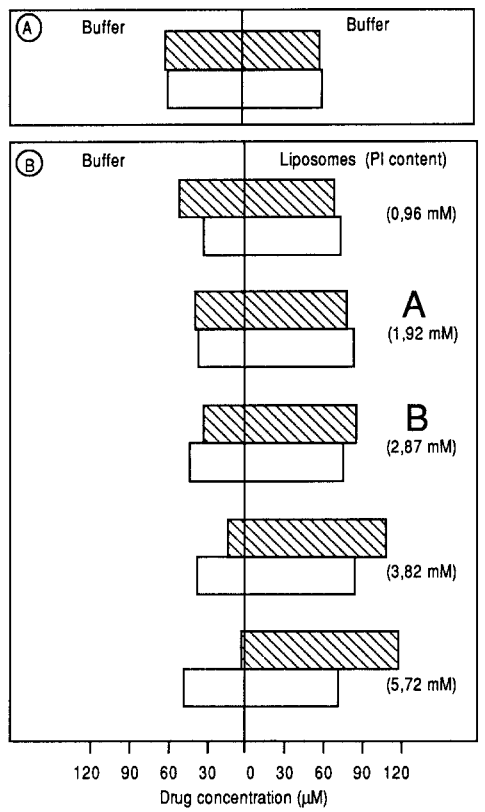

Fig. 4. Equilibrium dialysis of azithromycin (hatched bars) and gentamicin (open bars). I (A), dialysis against buffer (control); II (B), dialysis against small unilamellar vesicles of standard content in cholesterol (5.5 mol) and phosphatidylcholine (4 mol), and increasing amounts of phosphatidylinositol (from 1 to 6 mol) and decreasing amounts of sphingomyelin (from 6 to 1 mol). Drugs (initial concentration, 120 μM) in 40 mM Na acetate buffer were placed in the left chamber and liposomes (prepared in the same buffer at concentration of 15.7 mM in total lipids) were placed in the right chamber. The figures in brackets facing each series of bars show the actual phosphatidylinositol concentration; **A** and **B** refer to the two liposome compositions which have been used in the enzyme inhibition experiments described in Fig. 6. The abcissa shows the drug concentrations actually measured in the left chamber which represents the free drug (D_{free}), and the calculated concentrations of the total drug (bound and free drug; D_{total}) in the right chamber ($D_{total} = 120 - D_{free}$) at the end of the experiment.

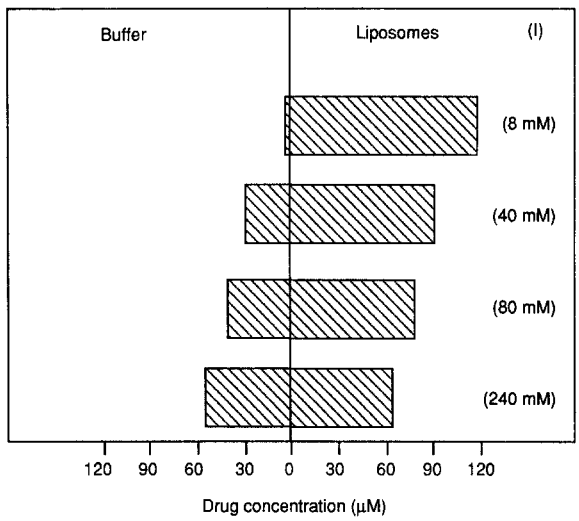

Fig. 5. Influence of the ionic strength on azithromycin binding to liposomes. Experiments were conducted with small unilamellar vesicles of the same composition as that shown Fig. 4A, but the drug was dissolved, and the liposomes initially prepared in a 4 mM Na acetate buffer, to which a suitable amount of concentred NaCl was added to obtain the ionic strengths indicated in parentheses on the right of each bar. Results are obtained and presented as in Fig. 4.

3.2. Binding of drugs to liposomes (small unilamellar vesicles)

The binding of the two antibiotics to liposomes was investigated by equilibrium dialysis using a constant initial drug concentration (120 μM) and small unilamellar vesicles with an increasing phosphatidylinositol content (from 1 to 6 mol per 11 mol of phospholipids; final concentrations, 0.96 to 5.72 mM), both placed in a 40 mM Na acetate buffer (ionic strength, 80 mM). Fig. 4 shows that the two drugs bind to liposomes under these conditions. Yet, whereas the proportion of bound azithromycin rose to 92% of the available drug when the phosphatidylinositol content was increased to the maximal value tested, the proportion of gentamicin bound never exceeded $\approx$ 30%.

Since Laurent et al. (1982) have demonstrated that the binding of gentamicin to negatively charged liposomes is largely impaired when the ionic strength of the buffer is increased, we have also investigated the influence of the ionic strength on the binding of azithromycin using liposomes with a low phosphatidylinositol content (final concentration, 1.92 mM), i.e., when binding was moderate. Liposomes were dispersed in a 4 mM Na acetate buffer to which NaCl was added in increasing concentrations, prior to addition of azithromycin, in order to bring the ionic strength (I) from 8 to 240 mM. Fig. 5 shows that the drug binding was considerably increased at low ionic strength (98% binding at $I = 8$ mM) whereas it was almost totally

suppressed at large ionic strength ($I = 240$ mM) under these conditions.

3.3. Inhibition of phospholipase A_1 activity (small unilamellar vesicles)

The inhibitory potency of azithromycin towards lysosomal phospholipase A_1 was investigated using small unilamellar vesicles containing 2 or 3 mol of phosphatidylinositol per 11 mol of total phospholipids. These compositions were choosen, because previous studies from our laboratory had indicated that the specific activity of lysosomal phospholipase A_1 increases 2–3-fold when the phosphatidylinositol content of the liposomes is in that range (Mingeot-Leclercq et al., 1988; see legend of Fig. 6 for specific activities of controls in the present experiments). This range also largely covers the variations seen in contents in acidic phospholipids of most membranes in vivo (Mc Murray and Magee, 1972), including the lysosomal membrane (Thinès-Sempoux, 1973). Parallel studies were run with gentamicin and spermine, and the results examined together with those of earlier studies using also gentamicin in comparison with DEH (Mingeot-Leclercq et al., 1988). Detailed results for azithromycin and gentamicin are shown in Fig. 6, and a summary of the data for all compounds is presented in Table 1.

All the polyaminated compounds caused a concentration-dependent inhibition of phospholipase A_1 activity [previous studies have shown that a hydrophilic monaminated compound like glucosamine is without effect up to 0.1 mM (Mingeot-Leclercq et al., 1988)]. In the present experiments, the activity of phospholipase A_1 was reduced by azithromycin and gentamicin to $\approx 20\%$ of its original value, after which no further inhibition was obtained (previous experience with this system shows that the level of residual activity varies from 20% as shown here to less than a few percent, depending on the batch of lysosomal extracts; the reason of this variability remains, however, unclear).

On a molar basis, azithromycin and gentamicin were essentially equipotent inhibitors under all conditions tested (see right inset in Fig. 6) whereas spermine and DEH were markedly less inhibitory (see Table 1). When considering equinormal concentrations, however, gentamicin and DEH were almost equipotent whereas azithromycin was a markedly stronger, and spermine a considerably weaker inhibitor.

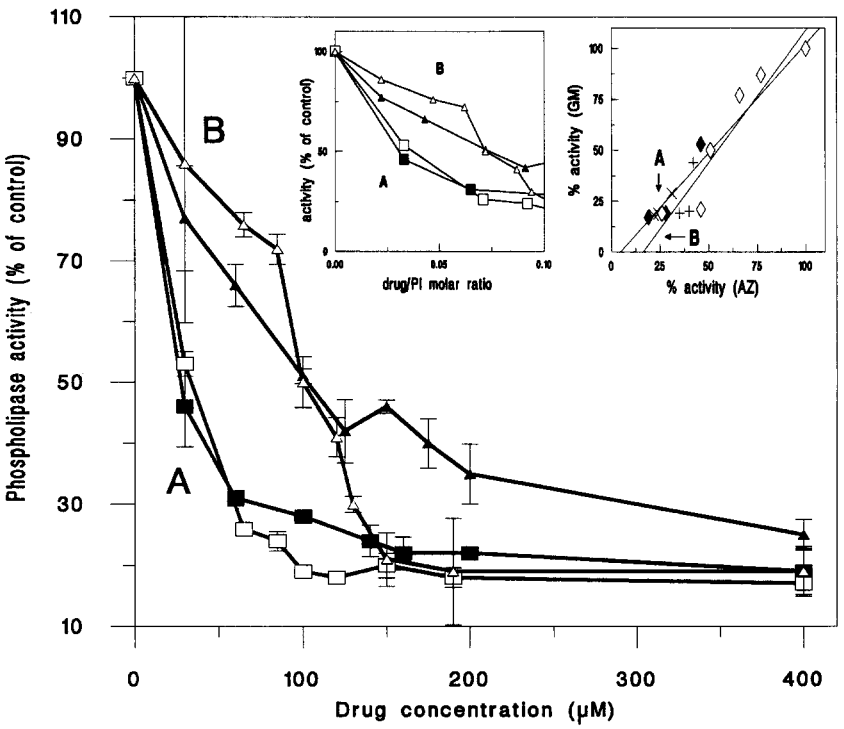

Fig. 6. Inhibition of the activity of lysosomal phospholipase A_1 by azithromycin (■, ▲) or gentamicin (□, △), towards phophatidylcholine included in small unilamellar vesicles. **A** and **B** refer to the two compositions of small unilamellar vesicles with the same content in phosphatidylcholine and cholesterol (4 and 5.5, respectively) but different phosphatidylinositol and sphingomyelin (2 and 5 mol, respectively, for vesicles **A**, and 3 and 4 mol, respectively, for vesicles **B**) to obtain membranes with distinct surface charges (these compositions are identical to those marked as **A** and **B** in Fig. 4). The final concentration of total lipids is 8 mM. Main graph: activity in percentage (± S.D.; $n = 3$) of that measured in the absence of antibiotic as a function of the final drug concentration in the assay mixture (specific activities of control: 15 and 29 mU/mg protein for conditions **A** and **B**, respectively). *Left inset*: same data, but activity is plotted as a function of the drug:phosphatidylinositol ratio (up to a drug concentration of 140 μM). *Right inset*: correlation between the activity of phospholipase A_1 in the presence of azithromycin and of gentamicin at equimolar concentrations; data presented are all actual values for azithromycin and are actual and intrapolated values for gentamicin); ◆ and ×: actual and intrapolated data from curves marked **A**, and ◇, + actual and intrapolated data from curves marked **B** in the main graph; the two lines correspond to linear regressions and their slopes are not statistically significant).

Table 1
Inhibitory potencies of polycations towards lysosomal phospholipase A_1

Agent (X, M_r) [b]	Concentration causing 50% inhibition [a]			
	PI vesicles content [c]	μM	μN	μg/ml
Azithromycin (2^+, 749)	2	34±3	68±6	26±2
	3	101±1	202±2	77±1
Gentamicin (5^+, 464 [d])	2	28±3	140±15	13.0±1
	3	102±10	510±50	47.4±5
Spermine (4^+, 348)	2	78±7	312±28	27±2
	3	163±10	652±40	56±3
DEH (2^+, 469)	2	86±5	172±10	40±2

[a] Values determined by intrapolation of data obtained in experiments conducted as described in Fig. 6. The figures are the mean ± S.D. of the values determined for each dose-response curve ($n = 3$).
[b] Figures under brackets show the number of cationic groups and the molecular weight of each agent (free base).
[c] Phosphatidylinositol content per 11 mol of phospholipids, corresponding to the sets of data marked A and B in Fig. 6, respectively.
[d] M_r of the C_{1a} component; other major components (C_1, C_2) have a M_r of 478 and show an inhibitory potency very similar to that of the C_{1a} component in this system (see Carlier et al., 1983).

Fig. 6 also shows that the inhibitory potencies of azithromycin and gentamicin were inversely proportional to the content of the liposomes in phosphatidylinositol (compare the sets of curves marked A and B in the main graph of Fig. 6; see also Table 1). This influence of phosphatidylinositol is still observed if enzyme activity is plotted as a function of the drug : phosphatidylinositol ratio up to a value of ≈ 0.1 (120 μM drug concentration; see left inset of Fig. 6).

At larger drug concentrations, the inhibition induced by azithromycin is less important than that caused by gentamicin when tested with vesicles with a large phosphatidylinositol content. Eventually, however, the maximal achievable inhibition (≈ 80%) was reached for both drugs at 400 μM. Although phosphatidylinositol exerted a negative effect on azithromycin- and gentamicin-induced inhibition, a minimal amount of this phospholipid (or of another negatively charged phospholipid) was necessary to elicit a significant inhibition. Thus, azithromycin caused only 23.5 ± 12.8% inhibition of phospholipase A_1 at a concentration of 400 μM when tested with small unilamellar vesicles containing only 0.2 mol of phosphatidylinositol per 11 mol of phospholipids [this effect was already reported and described in details for gentamicin (Mingeot-Leclercq et al., 1988; see also Piret et al., 1992)].

We also reported previously that gentamicin is not inhibitory towards lysosomal phospholipase A_1 activity when the latter is tested on detergent-dispersed phosphatidylcholine (mixed micelles) even if phosphatidylinositol is present (Laurent et al., 1982; Carlier et al., 1983). Thus, we repeated here the experiments described in Fig. 6, but added Triton X-100 (0.125%) and Na taurocholate (0.125%) to the liposomes-azithromycin mixture prior to the addition of the lysosomal enzyme preparation. No inhibition (activity was 99.3 ± 1.5% of that of control; $n = 3$) was detected at the largest azithromycin concentration tested (100 μM).

3.4. Aggregation and fusion of vesicles (large unilamellar vesicles)

3.4.1. Fluorescence dequenching studies

The measurement of the fluorescence dequenching of octadecylrhodamine B (R_{18}) is an established technique to study the fast mixing of lipids occurring during fusion of adjacent membranes (Hoekstra et al., 1984). We also used it previously to examine the slower exchange of lipids developing during membrane aggregation (Van Bambeke et al., 1995). This approach was, therefore, used with azithromycin. Melittin (Morgan et al., 1983) and gentam-

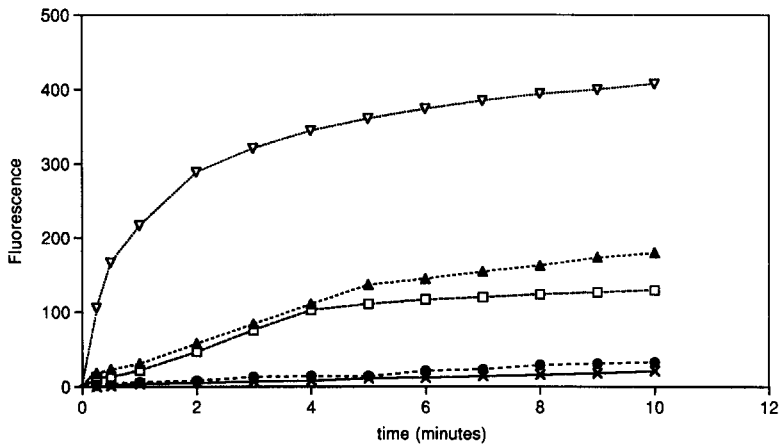

Fig. 7. Variation of the fluorescence (in arbitrary units) recorded after mixing labeled and unlabeled large unilamellar vesicles (ratio 1:4). The composition of the vesicles was similar to that used for obtaining the data marked as **B** in Figs. 4 and 6 (3 mol phosphatidylinositol per 11 mol of phospholipid; final total lipid concentration, 5 μM). (×) No treatment; addition of melittin (▽, 0.635 μM), spermine (□, 62.5 μM), gentamicin (▲, 62.5 μM), or azithromycin (●, 800 μM), yielding molar ratios of 0.7, 68.5, 68.5 and 880 to phosphatidylinositol, respectively. The fluorescence signal recorded prior to the drug addition (≈ 100 arbitrary units) has been substracted from all experimental points. Each data point shown is the mean of three independent experiments, with less than 10% variations (S.D. values have not been drawn for the sake of clarity).

icin and spermine (Van Bambeke et al., 1995) were used as positive controls for fusion and aggregation, respectively.

In a first series of experiments, we used liposomes containing the same lipids as those present in the small unilamellar vesicles used for the experiments described in Sections 3.2 and 3.3, with a phosphatidylinositol content set at 3 mol for 11 mol of total phospholipids (composition identical with that used to obtain the data marked B in Figs. 4 and 6). Results are shown in Fig. 7. In the absence of drug, the fluorescence signal increased at an exceedingly slow rate after mixing labeled and unlabeled liposomes. As anticipated (Van Bambeke et al., 1995), the addition of melittin immediately caused a marked increase in the fluorescence signal, which was already significant after 15 s and further progressed for ≈ 4–6 min until it eventually reached a plateau at ≈ 400 arbitrary units above control values. Addition of spermine or gentamicin (in a molar ratio of 62.5 to phosphatidylinositol) caused no immediate change but rather a slow increase for ≈ 4–6 min, after which the fluorescence signal remained almost stable at 120–180 arbitrary units over that of the control. This effect of spermine and gentamicin was dose-dependent and an increase in fluorescence signal could already be detected at a polycation/phosphatidylinositol ratio of 5.5 (Van Bambeke et al., 1995). In sharp contrast, addition of azithromycin, in a molar ratio to phosphatidylinositol of 880, did not cause any significant increase in the fluorescence of signal during the whole observation period (10 min) and not even after 30 min (data not shown). No effect of azithromycin was seen even if the phosphatidylinositol content of the liposomes was increased to 6 mol per 11 mol of phospholipids to obtain a maximal binding of gentamicin (data not shown).

Phosphatidylethanolamine is known to promote membrane fusion (Düzgünes et al., 1987), and is, therefore, often added to liposomes when testing the influence of potential fusogens. It also increases the extent of fluorescence dequenching occurring upon aggregation or fusion of liposomes (Van Bambeke et al., 1995). Our experiments were, therefore, repeated with large unilamellar vesicles containing phosphatidylethanolamine in a proportion ranging from 2.3 to 4 mol per 11 mol of total phospholipids. The expected increase in fluorescence signal was indeed observed with gentamicin and spermine (for melittin, see Van Bambeke et al., 1995) but azithromycin did not cause any detectable change over controls.

3.4.2. Light scattering spectroscopy

Changes in structural organization of liposomes, such as fusion or aggregation, can also be detected by examining their apparent size. This can be conveniently studied by light scattering spectroscopy, and the influence of azithromycin on this parameter was, therefore, investigated in comparison with gentamicin and spermine. Liposomes, prepared and treated as described for the experiments illustrated in Fig. 7, were, therefore, analyzed for apparent size before and after exposure to these compounds. As shown in Fig. 8, gentamicin and spermine not only caused a striking increase in the apparent diameter of the particles, but also made the vesicle population to appear very heterogenous. These two changes have been associated with the massive aggregation induced by these polycations in this system (Van Bambeke et al., 1995). In sharp contrast, azithromycin produced no detectable effect, even though its molar ratio with respect to phosphatidylinositol was 10 times greater.

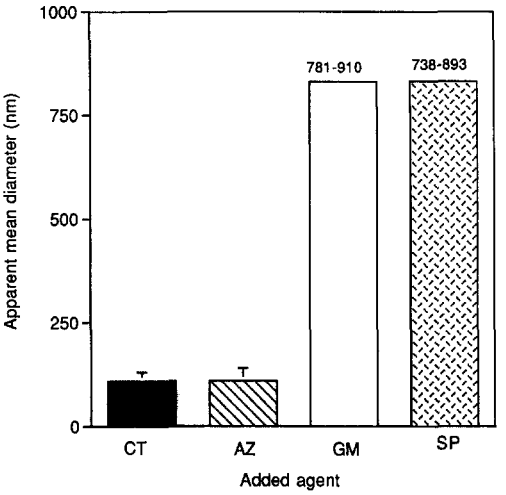

Fig. 8. Apparent mean diameter of the liposomes prepared and used in similar conditions as in the experiment described in Fig. 7. CT, control; AZ, addition of azithromycin; GM addition of gentamicin, SP, addition of spermine. The molar ratios of the polycations to phosphatidylinositol were 880, 68.5 and 68.5, respectively. Data obtained by light scattering spectroscopy on a Nanosizer (Coulter) instrument set on unimodal analysis. For control and azithromycin-treated liposomes, readings are given as nm ± S.D. (true unimodal populations). For gentamicin- and spermine-treated liposomes, multiple populations were detected by the instrument ('broad' S.D.) around the apparent mean value; the height of the blocks show those mean values and the figures in the graph show the size interval covering 95% of the corresponding vesicle population.

4. Discussion

Phospholipid storage disorders are induced in vivo and in cultured cells by a large array of drugs, with unrelated pharmacological actions and largely different structures but having all in common a cationic character and a capacity to interact with phospholipid bilayers (see Kodavanti and Mehendale, 1990, for review). Many of these drugs are lysosomotropic (see De Duve et al., 1974, for a definition of this term and for typical examples), so that lysosomes tend to be the first organelles in cells and tissues to display early and conspicuous signs of phospholipidosis. Ultrastructurally, these signs consist of the appearance of lamellated, osmiophilic inclusions (often referred to as 'myeloid bodies'; see typical examples in the

review of Lüllmann-Rauch (1979) and in the paper of Kosek et al. (1974)), and are very reminiscent of the alterations seen in genetic disorders of the lysosomal catabolism of polar lipids, such as the Niemann-Pick diseases (Lazarus et al., 1967).

The data presented in this paper, together with those of Shepard et al. (1992) and Carlier et al. (1994), which demonstrates that azithromycin accumulated by cells is associated to lysosomes, clearly indicates that azithromycin can be ranked among the group of cationic lysosomotropic drugs causing phospholipidosis.

4.1. Binding of azithromycin to negatively charged membranes

In most cases of phospholipidosis caused by cationic drugs, binding of the molecule to the phospholipids, and especially to the acidic ones, is often considered as a key initiating factor in the molecular sequence of events leading to the development of the cytological signs of phospholipidosis.

The dialysis experiments presented in this paper clearly show that azithromycin binds to phospholipid vesicles in direct proportion to their content in an acidic phospholipid. Although binding isotherms could not be obtained because of the lack of a sensitive and specific assay of azithromycin (radiolabeled drug with an acceptable purity could not be made available to us during the course of our experiments), examination of the data of Fig. 4 indicates that azithromycin probably displays a tighter binding than gentamicin. The latter showed K_d values ranging from 7 to 40 μM in the same system (Mingeot-Leclercq et al., 1990). Hydrophilic forces and charge neutralization are probably important in this binding since an increase in the ionic strength weakens it considerably. Yet, its tightness compared with gentamicin – which carries 5 ionizable amino-groups for only 2 in azithromycin – clearly suggests that additional forces, probably hydrophobic, are also important [see companion paper (Montenez et al., 1996)]. Whatever the mechanism, however, it seems reasonable to assume that azithromycin, transported and concentrated in the lysosomes of cultured fibroblasts, as indicated by the data of Gladue and Snider (1990) and Carlier et al. (1994), will tightly bind to acidic phospholipids present therein. This is all the more plausible, since Montenez (1996) showed that lysosomes of fibroblasts incubated with 50 mg/l (66 μM) azithromycin maintain an unchanged acidic pH ($\approx$ 5.2) in spite of the huge accumulation of this drug contrary to that reported for chloroquine (Ohkuma and Poole, 1978).

4.2. Mechanism of inhibition of lysosomal phospholipase A_1

The issue of the relationship between the binding of a cationic drug to phospholipids, the accumulation of undegraded phospholipids and the inhibition of lysosomal phospholipase activity has been extensively debated. Montenez (1996) found that phosphatidylcholine accounts for $\approx$ 60% of the phospholipids stored in excess in fibroblasts incubated as described in Fig. 3, and inhibition of the breakdown of that phospholipid is, therefore, of first importance in this context. Originally, Lüllmann et al. (1978) suggested that cationic drugs inhibit phospholipid degradation by forming complexes with them that are resistant to the action of phospholipases. Fowler and De Duve (1969) and Laurent et al. (1982) demonstrated that phospholipase A_1 is responsible for $\approx 2/3$ of the degradation of phosphatidylcholine in lysosomal extracts and, therefore, presumably also in lysosomes of living cells. In subsequent studies, Mingeot-Leclercq et al. (1988) showed that the activity of lysosomal phospholipases A_1 towards this phospholipid is critically dependent upon the negative charge carried by the bilayer and increases markedly when the content in acidic phospholipids is brought from 10 to 40% of the total lipids, i.e. within limits that are physiologically meaningful.

Binding of azithromycin to membrane bilayers and neutralization of their surface negative charges may, therefore, provide the molecular mechanism for the inhibition of phospholipase A_1 activity towards phosphatidylcholine breakdown. This mechanism was extensively studied with gentamicin and accounts for the paradoxical observations that an increase in phosphatidylinositol actually decreases the inhibitory potency of the drug in spite of its larger binding, because it restores a sufficiently large number of surface negative charges (see discussion in Mingeot-Leclercq et al. (1988) and Piret et al. (1992)). The similar behavior of azithromycin compared with gentamicin in this context indicates a similar mechanism.

Simple charge neutralization, however, does not explain all data, since the inhibitions caused by gentamicin, azithromycin, spermine and DEH clearly do not develop on an equinormal basis. We, therefore, need to postulate that binding and interactions of the drug with the phospholipids, which are likely to be different among these various agents, must also play an important role. It is also possible that the accessibilities of these 4 polycations to the membrane surface charges – and, therefore, their ability to neutralize them – are different. Membrane neutralization is, however, insufficient to cause a complete inhibition of the enzyme activity. This may result from the fact that a small but sizeable activity of phospholipase A_1 is detectable on neutral membranes (Mingeot-Leclercq et al., 1988).

An alternative explanation to charge neutralization, and involving membrane drug binding per se, is that the drug anchored at the membrane surface shields the substrate and causes inhibition by substrate depletion. This model was proposed for gentamicin by Hostetler and Jellison (1990). Yet, as explained in Mingeot-Leclercq et al. (1990) and Piret et al. (1992), this mechanism is simply not observed for gentamicin under the present experimental conditions.

Aggregation of membranes has also been proposed to explain phospholipase inhibition and is strongly suspected to occur in the cultured cells and in tissues in view of the cytological images of closely apposed layers ('myeloid bodies'), since it will obviously restrict the access of the enzyme to the substrate. Yet, as demonstrated here, azithromycin is not an aggregating agent but, nevertheless, is as inhibitory as gentamicin which causes massive aggregation. Moreover, inhibition with gentamicin is nearly maximal at a phosphatidylinositol-drug ratio of 0.1 whereas a ratio of 5.5 is necessary to induce detectable aggregation. This study, therefore, demonstrates that aggregation is probably not a likely mechanism for phospholipase inhibition induced by cationic drugs, at least under our experimental conditions. The data also suggests that myeloid bodies seen in the lysosomes of cultured cells are not accumulating there primarily because the drug would aggregate the membranes which end up or transit in lysosomes. The companion paper (Montenez et al., 1996) discusses the reason why azithromycin – although dicationic – may fail to aggregate negatively charged membranes.

Hostetler and co-investigators (Kubo and Hostetler, 1985, and the references cited in this paper) have also proposed that cationic drugs, such as chloroquine, could inhibit phospholipases by binding directly to the protein. This was not investigated directly here for azithromycin, but the fact that the drug does not inhibit lysosomal phospholipase A_1 activity when the liposomes in which phosphatidylcholine is included contain no or very little phosphatidylinositol, or if assays are made in the presence of detergents, would tend to disprove this possibility, since these modifications are more likely to affect the substrate (modification of surface charge or of lipid organization, respectively) or to prevent the drug binding to the bilayers than to affect the enzyme. A binding of the drug to the enzyme would indeed have caused an inhibition of the degradation of phosphatidylcholine included either in liposomes or in micelles.

4.3. Development of phospholipidosis in cultured cells

The in vitro data presented here on gentamicin- and azithromycin-induced inhibition of lysosomal phospholipases probably provides a clue for their effect on the cultured cells. Based on the data of Carlier et al. (1994), it can be estimated that the lysosomal concentration of azithromycin largely exceeds 1 mM in cells incubated with 50 mg/l (66 μM), i.e. much more than what is necessary to obtain a very large inhibition of phospholipase A_1 in vitro. [The same reasoning was applied for gentamicin in fibroblasts (see Aubert-Tulkens et al., 1979), even though much larger extracellular concentrations of this drug are needed because of its much lesser accumulation in fibroblasts (Tulkens and Trouet, 1978).] We do not know, however, if the residual activity observed in vitro truly translates into the maintenance of a corresponding catabolic activity in lysosomes or if it only reflects our inability to assay specifically for phospholipase A_1 activity in our system. We also need to examine the catabolism of other phospholipids, and the influence of azithromycin on other phospholipases.

The origin of the phospholipids stored in excess in cells treated with azithromycin cannot be ascertained at this stage. By analogy with gentamicin, however (see Aubert-Tulkens et al., 1979), a most plausible hypothesis is that these phospholipids are from endogenous origin and represent undigested components of cellular membranes sequestered in lysosomes by autophagy (see discussion in Lüllmann-Rauch, 1979). The organization of excess phospholipids in multiple, closely apposed layers could result from aggregation of these layers by the polycationic drugs, or simply by excess in accumulation of undegraded phospholipids in the limited space of lysosomes by impairment of their degradation. The latter hypothesis seems favored by the present data, since azithromycin is clearly not an aggregating agent.

4.4. Toxicological and pharmacokinetics implications

Administration of large doses of azithromycin to animals (200 mg/kg, $\approx$ 20 times the human therapeutic dose) for 4 days is associated with the appearance of myeloid bodies in liver, kidney and several other tissues (Shepard et al., 1992). Lower doses (20 mg/kg), however, did not induce this effect. An ultrastructural study of white blood cells from young adults treated with a total of 5 g over a period of 90 days also failed to reveal any significant intracellular deposition of lamellar material (Lambricht et al., 1994). As explained in details in Montenez (1996), early signs of phospholipidosis develop in fibroblasts when the apparent cellular concentration of the drug exceeds 1.3 mM. The administration of a single therapeutic dose of azithromycin (500 mg) will generate tissue concentrations up to 10–15 μM, giving a potential therapeutic index of $\approx$ 100. The situation will still be highly favorable for full treatments at conventional doses (typically, a total of 1.5 g in 3 or 5 days). Yet, binding of azithromycin to phospholipids in lysosomes could play a role in the maintenance of the sustained drug tissue levels observed in pharmacokinetic studies (Foulds et al., 1990), even if not eliciting detectable changes of these organelles.

The situation could become more critical and complex in case of prolonged administration of larger doses, such as those proposed for eradication of *Mycobacterium avium* in AIDS patients (up to 0.5 g/day for 30 days (Young et al., 1991) or 250 mg/day for several months). This point may well worth to be considered in pharmacovigilance studies.

Perhaps of more immediate value would be the use of the present data for the rational evaluation of other newly developed dicationic macrolide derivatives with still larger tissue accumulation, such as the 8a-aza-8a-homoeryth-

romycin (Pelak et al., 1993) or the promising ketolides (dibasic derivatives of erythromycin lacking the cladinose sugar moiety; Agouridas et al., 1995).

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References

Agouridas, C., A. Bonnefoy, K. Braham, K., P. Colette, M. Guitton, M., A. Hochet and J.F. Chantot, 1995, RU004: uptake by phagocytes, intracellular bioactivity and other immunomoludatory effects, in: 35th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, Abstract F175.

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

Bartlett, G.R., 1959, Phosphorus assay in column chromatography, J. Biol. Chem. 234, 466.

Bligh, E.G. and W.J. Dyer, 1959, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37, 911.

Bright, G.M., A.A. Nagel, J. Bordner, K.A. Desa, J.N. Dibrino, J. Nowakowska and F.C. Sciavolino, 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. 12, 1029.

Carlier, M.B., G. Laurent, P.J. Claes, H.J. Vanderhaeghe and P.M. Tulkens, 1983, Inhibition of lysosomal phospholipases by aminoglycoside antibiotics: comparative studies in vitro, Antimicrob. Agents Chemother. 23, 440.

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

Carlier, M.B., I. Garcia-Luque, J.P. Montenez, P.M. Tulkens and J. Piret, 1994, Accumulation, release and subcellular localization of azithromycin in phagocytic and non-phagocytic cells, Int. J. Tiss. React. 16, 211.

De Duve, C., T. De Barsy, B. Poole, A. Trouet, P.M. Tulkens and F. Van Hoof, 1974, Lysosomotropic agents, Biochem. Pharmacol. 23, 2495.

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

Düzgünes, N., K. Hong, P.A. Baldwin, J. Bentz, S. Nir and D. Papahadjopoulos, 1987, Fusion of phospholipid vesicles induced by divalent cations and protons. Modulation of phase transitions, free fatty acids, monovalent cations and polyamines, in: Cell Fusion, ed. A.E. Sowers (Plenum, New York, NY) p. 241.

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

Fowler, S. and C. De Duve, 1969, Digestive activity of lysosomes. III. The digestion of lipids by extracts of rat liver lysosomes, J. Biol. Chem. 244, 471.

Girard, A.E., D. Girard, A.R. English, T.D. Gootz, C.R. Cimokowski, J.A. Faiella and J.A. Retsema, 1987, Pharmacokinetic and in vivo studies with azithromycin (CP-62,993), a new macrolide with an extended half-life and excellent tissue distribution, Antimicrob. Agents Chemother. 31, 1948.

Gladue, R.P., G.M. Bright, R.E. Isaacson and M.F. Newborg, 1989, In vitro and in vivo uptake of azithromycin (CP-62,993) by phagocytic cells: possible mechanism of delivery and release at sites of infection, Antimicrob. Agents Chemother. 33, 277.

Gladue, R.P. and M.E. Snider, 1990, Intracellular accumulation of azithromycin by cultured human fibroblasts, Antimicrob. Agents Chemother. 34, 1056.

Grove, D.C. and W.A. Randall, 1955, in: Assay Methods of Antibiotics, eds. D.C. Grove and W.A. Randall (Medical Encyclopedia, New York, NY) p. 14.

Hoekstra, D., T. De Boer, K. Klappe and J. Wilschut, 1984, Fluorescence method for measuring the kinetics of fusion between biological membranes, Biochemistry 23, 5675.

Hope, M.J., M.B. Bally, G. Webb and P.R. Cullis, 1985, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta 812, 55.

Hostetler, K.Y. and E.J. Jellison, 1990, Inhibition of kidney phospholipase A1 by aminoglycosides is a novel variant of substrate depletion inhibition, J. Pharmacol. Exp. Ther., 188.

Kirst, H.A. and G.D. Sides, 1989a, New directions for macrolide antibiotics: structural modifications and in vitro activity, Antimicrob. Agents Chemother. 33, 1413.

Kirst, H.A. and G.D. Sides, 1989b, New directions for macrolide antibiotics: pharmacokinetics and clinical efficacy, Antimicrob. Agents Chemother. 33, 1419.

Kodavanti, P. and H.M. Mehendale, 1990, Cationic amphiphilic drugs and phospholipid storage disorder, Pharmacol. Rev. 42, 327.

Kosek, J.C., R.I. Mazzer and M.J. Cousins, 1974, Nephrotoxicity of gentamicin, Lab. Invest. 30, 48.

Kubo, M. and K.Y. Hostetler, 1985, Mechanism of cationic amphiphilic drug inhibition of purified lysosomal phospholipase A1, Biochemistry 24, 6515.

Lambricht, P., P.J. Courtoy, P. Maldague, S. Ibrahim, P.M. Tulkens and R.R. Daniel, 1994, Safety of azitromycin and erythromycin in long-term treatments: analysis of phospholipiduria and phospholipidosis in white blood cells, in: Future Trends in Chemothery (Geneva, Switzerland) Abstract 91.

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

Lazarus, S., S. Vethamany, L. Schneck and B.V. Volk, 1967, Fine structure and histochemistry of peripheral blood cells in Niemann-Pick disease, Lab. Invest. 17, 155.

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

Lüllmann, H., R. Lüllmann-Rauch and O. Wassermann, 1978, Lipidosis induced by amphiphilic cationic drugs (commentary), Biochem. Pharmacol. 27, 1103.

Lüllmann-Rauch, R., 1979, Drug induced lysosomal storage disorders, 1979, in: Lysosomes in Applied Biology and Therapeutics, Vol. 6, eds. J.T. Dingle, P.J. Jacques and I.H. Shaw (Elsevier, Amsterdam) p. 49.

Mazer, N.A., M.C. Carey, R.F. Kwasnick and G.B. Benedek, 1979, Quasielastic light scattering studies of aqueous biliary lipid systems. Size, shape and thermodynamics of bile salt micelles, Biochemistry 18, 3064.

Mc Murray, W.C. and W.L. Magee, 1972, Phospholipid metabolism, Annu. Rev. Biochem. 41, 129.

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

Mingeot-Leclercq, M.-P., A. Schanck, M.F. Ronveaux-Dupal, M. Deleers, R. Brasseur, J.M. Ruysschaert and P.M. Tulkens, 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.

Mingeot-Leclercq, M.-P., J. Piret, R. Brasseur and P.M. Tulkens, 1990, Effect of acidic phospholipids on the activity of lysosomal phospholipases and on their inhibition by aminoglycoside antibiotics. I. Biochemical analysis, Biochem. Pharmacol. 40, 489.

Mingeot-Leclercq, M.-P., R. Brasseur and A. Schanck, 1995, Molecular parameters involved in aminoglycosides nephrotoxicity, J. Toxicol. Environ. Health 44, 263.

Montenez, J.P., B.K. Kishore, P. Maldague and P.M. Tulkens, 1994, Leupeptin and E-64, inhibitors of cysteine proteinases, prevent gentamicin-induced lysosomal phospholipidosis in cultured rat fibroblasts, Toxicol. Lett. 73, 201.

Montenez, J.P., 1996, Etude des surcharges lysosomiales induites par l'azithromycine dans des cellules en culture: effets sur les phospholipides et le cholestérol cellulaires, in: Thesis (Université Catholique de Louvain, Bruxelles, Belgium) pp. 185.

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

Morgan, C.G., H. Williamson, S. Fuller and B. Hudson, 1983, Melittin induces fusion of unilamellar phospholipid vesicles, Biochim. Biophys. Acta 732, 668.

Ohkuma, S. and B. Poole, 1978, Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents, Proc. Natl. Acad. Sci. USA 75, 3327.

Pelak, B.A., L.S. Gerckens and H. Krapp, 1993, Tissue distribution and pharmacokinetics of novel azalide 8a-aza-8a-homoerythromycin derivatives, in: 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, LA, Abstract 428.

Piret, J., B.K. Kishore and P.M. Tulkens, 1992, Effect of substrate organization on the activity and on the mechanism of gentamicin-induced inhibition of rat liver lysosomal phospholipase A1, Biochem. Pharmacol. 43, 895.

Shepard, R.M., D.E. Amacher, M.A. Mullins, R.A. Ferraina, C.V. Higgins, A.B. Jakowski and W.B. Milisen, 1992, Mechanism of azithromycin tissue uptake and accumulation: subcellular localization of azithromycin in rat liver, in: 1st International Conference on the Macrolides, Azalides and Streptogramins, Santa Fé, NM, Abstract 217.

Steigbigel, N.H., 1995, Macrolides and clindamycin, in: Principles and Practice of Infectious Diseases, 4th Ed., eds. G.L. Mandell, J.E. Bennett and R. Dolin (Churchill Livingstone, New York, NY) p. 334.

Thinès-Sempoux, D., 1973, A comparison between the lysosomal and the plasma membrane, in: Lysosomes in Biology and Pathology. Vol 3, eds. J.T. Dingle and H.B. Fell (Elsevier, Amsterdam) p. 278.

Trouet, A., 1974, Isolation of modified liver lysosomes, Methods Enzymol. 31, 323.

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

Tulkens, P. and A. Trouet, 1978, The uptake and intracellular accumulation of aminoglycosides antibiotics in lysosomes of cultured fibroblasts, Biochem. Pharmacol. 27, 415.

Udenfriend, S., S. Stein, P. Böhlen, W. Dairman, W. Leimgruber and M. Weigele, 1972, Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range, Science 178, 871.

Van Bambeke, F., M.-P. Mingeot-Leclercq, A. Schanck, R. Brasseur and P.M. Tulkens, 1993, Alterations in membrane permeability induced by aminoglycoside antibiotics: studies on liposomes and cultured cells, Eur. J. Pharmacol. 247, 155.

Van Bambeke, F., P.M. Tulkens, R. Brasseur and M.-P. Mingeot-Leclercq, 1995, Aminoglycoside antibiotics induce aggregation but not fusion of negatively-charged liposomes, Eur. J. Pharmacol. 289, 321.

Villa, P., D. Sassella, M. Corada and I. Bartosek, 1988, Toxicity, uptake, and subcellular distribution in rat hepatocytes of roxithromycin, a new semisynthetic macrolide, and erythromycin base, Antimicrob. Agents Chemother. 32, 1541.

Young, L.S., L. Wiviott, M. Wu, P. Kolonoski, R. Bolan and C.B. Inderlied, 1991, Azithromycin for treatment of mycobacterium avium intracellulare complex infection in patients with AIDS, Lancet 338, 1107.