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INTERACTIONS OF AMIODARONE WITH MODEL MEMBRANES AND AMIODARONE-PHOTOINDUCED PEROXIDATION OF LIPIDS

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Abstract—The potent antiarrhythmic drug, amiodarone (AMIO) exhibits phototoxicity, which is thought to be related to its interaction with biological membranes. We report here a spectroscopic study of the interactions of this drug with phosphatidylglycerol (PG) and phosphatidylcholine (PC) liposomes used as membrane model systems. A linear increase in absorbance at 300 nm was observed with increasing addition of AMIO to dimyristoyl-DL-PC (DMPC) liposomes over all the drugs–lipid molar ratio (*Ri*)s tested. In contrast, in the dimyristoyl-DL-PG (DMPG) liposomes, there was a dramatic increase in absorbance at values of *Ri* above unity. Light scattering by DMPG liposomes at 350 nm increased with increasing AMIO concentration up to a $Ri = 1$, and then decreased with increasing drug concentration. Such changes were not observed with the DMPC liposomes. Moreover, addition of AMIO changed the fluorescence polarization rate of 1,6-diphenyl 1,3,5-hexatriene embedded in these liposomes. It reduced the rate below the phase transition temperature (*Tt*) of the lipid, but increased it above this temperature. These effects on the lipidic phases observed at low *Ri* were more pronounced on the DMPG than on the DMPC liposomes. The strong interactions of AMIO with phospholipids, especially the acidic ones, were confirmed by liposome size determinations. All these data strongly suggest that the drug was incorporated in the core of the lipid bilayers. Such a penetration would favor a drug-photoinduced peroxidation of lipids. Indeed, UV irradiation of AMIO–DOPG mixtures led to the disappearance of the unsaturated fatty acids of phospholipids, checked by gas chromatography measurements, which was correlated with the amount of oxygen consumed. This showed that AMIO did photosensitize phospholipid peroxidation.

Amiodarone (AMIO§) (Fig. 1) is a widely used cardiac antiarrhythmic drug [1–3]. However, numerous undesirable actions have been described for this drug, including actions on blood lipids [4], lipid metabolism [5], thyroid hormone metabolism [6] and the lung [7]. This drug also preferentially accumulates in liver, lung and adipose tissue where some ultrastructural changes have been described when the drug is administered for a long period [8, 9]. Moreover, it has been reported to induce photosensitivity in patients treated for a prolonged period of time with this drug [10]. This photosensitivity is characterized by an intense burning, erythema and swelling of the exposed area, followed in some cases by the appearance of a blue-grey pigmentation [10–12]. Histological and histochemical investigations of the pigmented skin have shown the presence of lipofuschinic and melanic granules in macrophages of the superficial dermis [13]. The accumulation of iodide in skin pigments has also been mentioned [14].

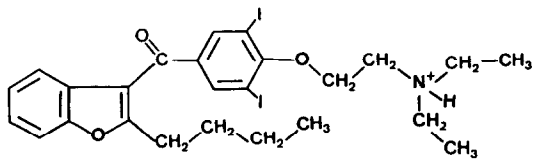

Fig. 1. Chemical structure of AMIO.

At a molecular level, photosensitization has been thought to be due mainly to membrane damage [15, 16]. This assumption is supported by the observed photohemolytic action of AMIO *in vitro* [15]. It has been demonstrated that under irradiation, AMIO undergoes a radical dehalogenation, and in the presence of oxygen promotes formation of singlet oxygen [17, 18]. Short-lived species such as radical or singlet oxygen may provoke damage in membranes which could account, at least in part, for the phototoxic properties of AMIO.

The impact of such processes will also depend on the localization of the drug in cells. AMIO is composed of a large hydrophobic part and a small hydrophilic tail (Fig. 1). This amphiphatic character explains the high affinity of AMIO for membranes.

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§ Abbreviations: AMIO, amiodarone; DMPG, dimyristoyl-DL-phosphatidylglycerol; DMPC, dimyristoyl-DL-phosphatidylcholine; DOPG, dioleoylphosphatidylglycerol, DOPC, dioleoylphosphatidylcholine; DPH, 1,6-diphenyl 1,3,5-hexatriene; SUV, small unilamellar vesicle; MLV, multilamellar vesicle, DMF, dimethyl formamide.

As it is positively charged at physiological pH, it is liable to interact with acidic molecules. AMIO penetrates tissues rapidly after administration [19, 20] and has a high lipid or membrane partition coefficient [21, 22]. It forms micelles [23, 24] and monolayers at the air–water interface [25], and strongly interacts with lipid bilayers or vesicles used as membrane model systems [26–29].

In the present study, we examined the incorporation of AMIO into liposomes and the subsequent AMIO-photoinduced oxidation of liposomal lipids. The interactions of AMIO with pure acidic (DMPG) or zwitterionic (DMPC) liposomes were investigated by UV spectroscopy, light scattering and fluorescence polarization. The AMIO-photosensitized oxidation of DOPG small unilamellar vesicles was then followed by oxygraphy, and the analysis of the oxidized phospholipids was evaluated by quantitative gas chromatography.

MATERIALS AND METHODS

Chemicals. AMIO or 2-butyl-3(4-(2-diethylaminoethoxy)-3′,5′-diiodobenzoyl)-benzofuran hydrochloride was a generous gift from Labaz-Sanofi Laboratories (Montpellier, France). DMPG, DMPC, DOPG, DOPC and DPH were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The purity of the phospholipids was checked by TLC.

Phospholipid dispersions. The UV and light scattering experiments were carried out using small unilamellar vesicles (SUVs) prepared according to Huang [30]. Dried lipids were suspended in a 10 mM NaCl solution by vortex agitation and sonicated in a bath until a clear solution was obtained.

For the photolysis experiments, SUV dispersions were prepared as described above except that sonication was carried out using a titanium probe for 3 min in ice, followed by centrifugation at 3000 *g* to eliminate titanium particles and multilamellar vesicles (MLVs). The whole experiment was performed under a stream of nitrogen.

For polarization and nanosizer experiments, we used MLVs prepared according to Bangham *et al.* [31].

The drug concentration depended on the technique and was specified below in each case.

UV and light scattering. For the UV and light scattering experiments, the samples were prepared by stepwise addition of 2 μL of AMIO solution (2.5×10^{-3} M in DMF) to 2 mL of a SUV dispersion (lipid concentration: 2.5×10^{-5} M). Correspondingly, 2 μL of DMF were added stepwise to the reference cuvette containing 2 mL of a liposomal dispersion (2.5×10^{-5} M). One experiment was also carried out by coevaporation of 2 mL of a lipid solution (2.5×10^{-5} M) with 2 μL of a solution of AMIO in DMF (2.5×10^{-3} M) followed by dilution with 2 mL of NaCl (10 mM).

The absorbance of SUV dispersions was measured at 300 nm with a Beckman 5260 spectrophotometer in a temperature-controlled cuvette holder equipped with a magnetic stirrer. Light scattering was measured using an Aminco SPF 500 fluorimeter at an excitation and emission wavelength of 350 nm. Light scattered by liposomal solutions containing AMIO was measured at 90° of the exciting light beam.

Fluorescence polarization. The experiments were carried out in a T-format automatic apparatus constructed in the laboratory and connected to a microcomputer. The excitation channel consisted of a light source (mercury or xenon arc lamp), a shutter, a monochromator, and a rotating polarizer (Glan prism), polarizing the incident light alternately in the vertical (fluorescence polarization measurement) or horizontal planes (determination of the relative sensitivity of the two detection systems). The detection system comprised two independent channels measuring the vertical I_V and the horizontal I_H components of the fluorescence emission simultaneously. Samples were placed in a closed, stirred and thermostated housing system. The temperature could be regulated using a Peltier element from 4° up to 60°. Quartz optics were used to allow measurements in the UV region. The fluorescence polarization rate is given as

$$P = (I_V - I_H)/(I_V + I_H).$$

The liposomal suspension contained lipids at a concentration of 7×10^{-5} M in 0.1 M phosphate buffer containing 10^{-6} M EDTA (pH = 7.4), 2×10^{-6} M DPH and different amounts of AMIO. Absorbance of the sample at 400 nm was kept below 0.1 to reduce the light scattering due to liposomes.

Size determination of liposomes. A Coulter N4MD nanosizer was used to determine the size of the liposomes. A statistical size distribution processor (SPD analysis) provided a histogram of the size distribution of the particles in the sample studied. These measurements were performed with MLV at a lipid concentration of 5×10^{-4} M, the drug concentration varying from 10^{-4} M to 7.5×10^{-4} M ($0.2 < Ri < 1.5$).

Irradiation and oxygraphic measurements. Irradiation was carried out with a Muller illumination device equipped with a high pressure mercury lamp (Osram), 200 Watts, using a quartz fiber (5 mm diameter, 50 cm long) focused on the oxygraphic cell. This Pyrex glass cell contained 1.5 mL of solution in direct contact with a YSI Clark electrode. The oxygraphic cell was thermostated at 37°. A 10 mM solution of NaCl bubbled with oxygen or argon for 10 min was used as a control. The pyrex glass of the cell cut off all the radiations < 300 nm.

Measurements of oxygen uptake by DOPG and DOPC liposomes in the presence or absence of AMIO, with or without irradiation, were performed on liposomes of 1–1 (mol–mol) mixtures of DMPG and DOPG, or DMPC and DOPC. It should be noted that in these experiments, DMPG or DMPC was used as internal standard as the saturated fatty acids in these phospholipids were found not to undergo oxidation under irradiation in either the presence or absence of AMIO. All experiments were carried out at 37°.

A value of 0.4 was selected for *Ri* to avoid the "bursting effect" of AMIO on lipid vesicles. Liposomal suspension (150 mL) with or without AMIO was placed in the cell filled with an O_2-saturated NaCl solution (1.5 mL). The final concentrations in the oxygraphic cell were

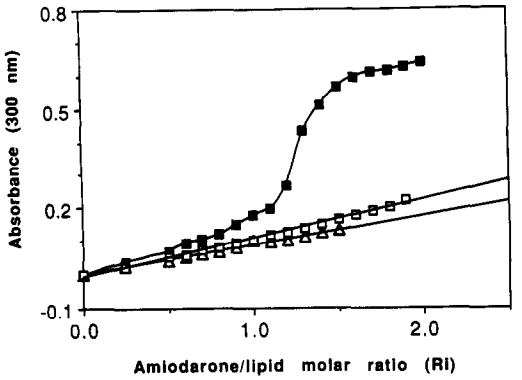

Fig. 2. Change in absorbance at 300 nm of AMIO in DMPG (■) or DMPC (□) liposomes or in DMF solution (△) as a function of the amiodarone–lipid molar ratio, *Ri*.

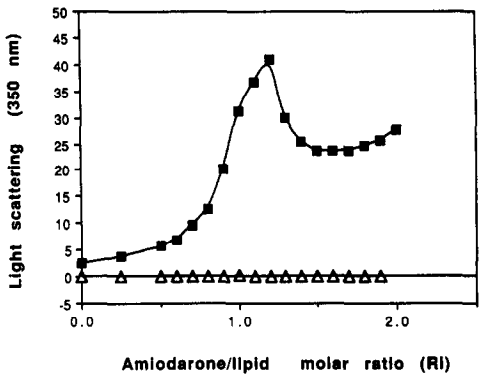

Fig. 3. Light scattering at 350 nm by AMIO in DMPG (■) or in DMPC (△) liposomes.

3.4×10^{-4} M for AMIO, 4×10^{-4} M for DMPG and 4×10^{-4} M for DOPG (1–1 DMPG–DOPG mixture) or 8×10^{-4} M when DMPG was used alone with AMIO. The samples were irradiated from 0 to 30 min.

Gas chromatography analysis. In order to measure the change in the proportion of unsaturated fatty acids in phospholipids after oxidation, the previously irradiated samples were lyophilized and treated with methanol/HCl, for 1 hr at 75°. The fatty acid methyl esters were then extracted with petroleum ether and analysed by gas chromatography using a Perkin–Elmer 8310B apparatus equipped with a 20% DEGS column (2.5 m) at 180°.

RESULTS

UV spectroscopy

The interaction of AMIO with liposomes was first studied by monitoring the absorbance of various AMIO–lipid mixtures containing increasing amounts of AMIO. The concentrations of AMIO ranged from 3×10^{-6} to 4×10^{-5} M corresponding to a range of 0.15–2 in *Ri*. These experiments were carried out at 300 nm, a wavelength at which AMIO was absorbing alone and only a slight diffusion by liposomes was recorded. As can be seen in Fig. 2, three patterns of change were observed when AMIO was added to DMPG liposomes: (1) as *Ri* increased from 0 to 1.15, the absorbance varied linearly with increasing *Ri* according to Beer-Lambert's law. These changes were comparable to those observed when a solution of AMIO in DMF was added to an aqueous solution (10 mM, NaCl). (2) as *Ri* increased from 1.15 to 1.3, there was a dramatic increase in absorbance. (3) for $Ri > 1.3$, a smaller and linear change in absorbance was observed. The slope of the straight line was similar to that observed for $Ri < 1.15$. Identical results were obtained when SUVs were directly prepared from mixtures of the drug and phospholipids.

In contrast, absorbance at 300 nm varied linearly with drug concentration in the DMPC liposomes over the whole range of *Ri* (0–2) examined, and no abrupt change in the optical properties of the system was observed.

The same differences in behavior were observed between the DOPG and DOPC liposomes.

Light scattering

We also examined the changes in light scattered by the same liposomal preparations in relation to AMIO concentration (or *Ri*). As shown in Fig. 3, light scattering reached a maximum for $Ri = 1.1$ for the DMPG–AMIO systems, whereas it remained constant at all drug concentrations in the DMPC–AMIO systems. The same difference in behavior was observed between the DOPG and DOPC liposomes.

It should be noted that the maximum light scattering observed for DMPG and DOPG was observed at the same value of *Ri* as that at which there was a marked change in the absorbance of these systems.

Taken together, UV spectroscopy and light scattering data indicated marked preference of the cationic drug AMIO for acidic phospholipids (PG) as compared to zwitterionic lipids (PC), regardless of the nature of the acyl chains (saturated or unsaturated) present in these molecules. The fact that with both methods dramatic changes in the recorded signal were observed for *Ri* just above unity strongly suggested that electrostatic forces were involved in these drug–lipid interactions. These observations also indicated that these interactions might result in extensive modifications of the structure of liposomes.

Fluorescence polarization

Figure 4 shows the effects of AMIO on the polarization rate *P* of the probe DPH embedded in MLVs of DMPG (Fig. 4a) or DMPC (Fig. 4b) for different *Ri* values and with varying temperature.

In the absence of drug (full line), the transition temperatures (*Tt*) of DMPG and DMPC were 24.6°C and 24.3°C, respectively, in agreement with previous reports.

First, when AMIO was added to acidic DMPG liposomes (Fig. 4a), we observed a dose-dependent decrease of *P* below *Tt*, i.e. a fluidizing effect of the

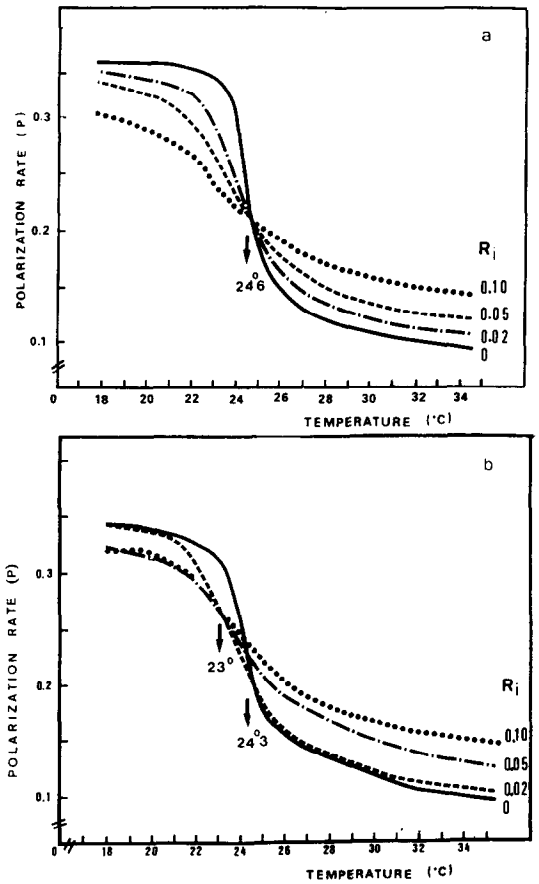

Fig. 4. (a) Fluorescence polarization rate of DPH in DMPG liposomes in the absence (full line) or presence (dotted lines) of various amounts of AMIO (*Ri* ranging from 0 to 0.1). (b) Fluorescence polarization rate of DPH in DMPC liposomes in the absence (full line) or presence (dotted lines) of various amounts of AMIO (*Ri* ranging from 0 to 0.1).

drug, and an increase of *P* above the phase transition temperature, i.e. a rigidifying effect of the drug. This effect was accompanied by an alteration in the absolute value of *Tt*, which is in agreement with the results of Chatelain *et al.* [27].

Second, from Fig. 4b, it can be seen that AMIO interacted with DMPC liposomes in the fluid phase (above *Tt*), and to a lesser extent when the lipid was in the gel phase (below *Tt*). *Tt* was significantly reduced (from 24.3° to 23°) in all cases.

Third, *P* could not be determined at values of *Ri* above 0.1 for both lipids, indicating a marked disordering effect of the drug on lipid molecular packing.

Influence of AMIO on the size and structure of liposomes

The light scattering (Fig. 3) and fluorescence polarization studies (Fig. 4a and b) indicated that AMIO, when interacting with lipids, induced marked changes in the physical state of the DMPG liposomes and to a lesser extent of the DMPC liposomes. To confirm that the drug did in fact modify liposome structure, we measured the mean size of these liposomes as a function of AMIO concentration at two temperatures: 18° and 27°. The results obtained with the nanosizer are shown in Table 1. Several observations can be made from these results, as follows.

First, the DMPG liposomes were smaller than the DMPC liposomes (1850 ± 500 instead of 3790 ± 1100 nm) when lipids were in the gel phase (at 18°). The DMPG and DMPC liposomes were also smaller at 27° than at 18°.

Second, a "destructuring" effect of AMIO corresponding to a decrease in the size of the liposomes was observed with both classes of lipids. Nevertheless, it was more marked and appeared at lower drug concentrations in the DMPG liposomes as compared with the DMPC liposomes. At 18°, there was a change in the DMPG MLVs at $Ri = 0.5$, but only at $Ri = 1.5$ for the DMPC MLVs.

Third, 66% of the DMPG MLVs were markedly smaller (145 nm ± 36) above *Tt* (27°) for $Ri = 1.5$. They were 10-fold smaller than the untreated liposomes. For DMPC MLVs at the same temperature and for $Ri = 1.5$, 89% of the total population was observed around 1200 nm (i.e. 1.6-fold smaller than controls).

Unfortunately, we were unable to measure the absolute size of the liposomes by negative staining electron microscopy, probably because the step which included the dehydration of the sample excluded the big liposomes. In fact, the mean sizes obtained were consistently smaller than those measured in the N4MD nanosizer for all lipids. However, the same qualitative "destructuring" effect was observed at drug to lipid ratios above unity. Nevertheless, this technique showed that the vesicles retained a vesicular shape after interaction with AMIO.

These results demonstrate that interaction of AMIO with liposomes at $Ri > 1$ effectively burst the liposomes, producing much smaller vesicular structures. This effect was more marked with the DMPG than with the DMPC liposomes.

Lipid peroxidation

Oxygen uptake. To measure photoinduced lipid peroxidation, DOPG–DMPG and DOPC–DMPC liposomes (DOPG: 6×10^{-7} mol, DMPG: 5.87×10^{-7} mol) were irradiated by UV light ($\lambda > 300$ nm) in an oxygraphic cell in the presence or absence of AMIO. DMPG was not modified when irradiated with AMIO under these conditions (data not shown) and was used as a standard in the gas chromatography measurements. As shown in Fig. 5, when irradiated for 30 min in the presence of AMIO, DOPG–DMPG liposomes consumed 95% of the total oxygen (6.1×10^{-7} mol) available in the cell. This is to be compared to the 6% consumed by the lipid and the 47% by the drug when irradiated alone. Control experiments showed that lipids alone, AMIO in DMF solution or AMIO in DMPG liposomes consumed little oxygen in the absence of irradiation (around 5%).

The oxidation rate of DOPG (under irradiation, in the presence of AMIO) was estimated from

Table 1. Effect of AMIO on the mean size of DMPG and DMPC liposomes at 18° and 27°

	DMPG				DMPC			
	18°		27°		18°		27°	
Ri	(%*)	(nm†)	(%*)	(nm†)	(%*)	(nm†)	(%*)	(nm†)
	8	37 ± 4			4	41 ± 10		
0	8	297 ± 71	17	163 ± 53	4	753 ± 240	2	265 ± 66
	84	1850 ± 500	83	1420 ± 370	92	3790 ± 1100	98	2020 ± 570
	17	50 ± 12						
0.2	11	501 ± 190	22	160 ± 41	9	764 ± 200	3	170 ± 69
	73	1810 ± 230	78	1120 ± 130	91	4030 ± 530	97	1930 ± 670
0.5	21	308 ± 80	22	148 ± 36	16	747 ± 150	6	320 ± 45
	79	1080 ± 200	78	1120 ± 140	84	3980 ± 650	94	1960 ± 450
	63	24 ± 3						
1	13	319 ± 53	12	264 ± 66	23	688 ± 160	12	392 ± 100
	24	1690 ± 260	88	1220 ± 220	77	3400 ± 880	88	1810 ± 230
1.5			66	145 ± 36	12	484 ± 99	11	254 ± 66
	100	340 ± 99	34	750 ± 88	88	2050 ± 520	89	1220 ± 230
F		5		10		18		1.6

* Percentage of the total population
† Means ± SD.
F, Factor of diminution of the size.

Table 2. Correlation between the amount of oxygen (moles) consumed by DMPG–DOPG liposomes irradiated in the presence of AMIO and the amount of unsaturated fatty acids (moles) which were lost during the oxidation process

Irradiation time (min)	Consumed oxygen ($\times 10^7$ mol)	Loss of unsaturated fatty acids ($\times 10^7$ mol)	% of lost unsaturated fatty acids*
0	0	0	0
4	1.15	0.19	1.6
6	1.28	1.95	16.3
10	2.27	2.44	20.3
20	3.80	4.05	33.7
30	5.79	5.37	44.7

* Measured from gas chromatograms by reference to saturated fatty acids.

the oxygen consumed by DOPG–DMPG–AMIO mixture after correction for the oxygen consumed by AMIO alone (in DMF or in DMPG liposomes). As can be seen in Fig. 5, the so calculated oxidation rate of DOPG in the presence of AMIO (corrected line), was far higher than that observed in the absence of AMIO. This indicates that the oxidation of DOPG was largely due to photosensitization by AMIO. It should be noted that this photosensitizing effect was probably underestimated. Indeed, the oxidation rate of AMIO itself under irradiation is expected to be lower in the presence than in the absence of unsaturated lipids because, in the presence of unsaturated lipids, both drug and unsaturated lipid molecules compete for oxygen.

Gas chromatography determinations. Oxidation of unsaturated fatty acids usually yields short chain and highly polar products. These oxidation products are water soluble and when working in water, as was the case in the present study, they are difficult to isolate for further analysis. One possible way to monitor fatty acid oxidation is to analyse, by gas chromatography and using an internal reference, the proportion of unsaturated fatty acids which remains present in phospholipids after oxidation and methanolyse have been performed. The differences which are observed in the gas chromatograms obtained before and after oxidation give the proportion of unsaturated fatty acids which have been lost during the oxidation process. Accordingly, fatty acid content was analysed in phospholipid mixtures previously irradiated in the presence or in the absence of AMIO. First, control experiments showed that in both cases, the saturated fatty acid content was not altered. Unsaturated fatty acids (oleic acid: 12×10^{-7} mol) were not significantly oxidized when DOPG liposomes were irradiated in the absence of AMIO (2% after 30 min). In contrast, strong alterations in the saturated/unsaturated fatty acid ratios were observed when these liposomes were irradiated in the presence of AMIO. As can be seen in Table 2, the amount of unsaturated fatty acids which disappeared after irradiation of DOPG in the presence of AMIO increased with duration of

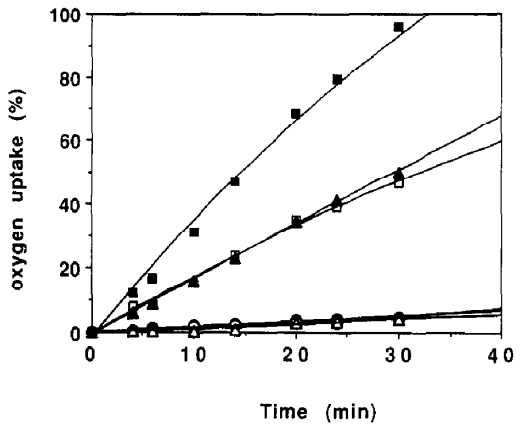

Fig. 5. Oxygen uptake (%) by DOPG–DMPG liposomes. Irradiated solutions: DOPG+DMPG liposomes alone (△), DOPG+DMPG liposomes with AMIO (■), DMPG liposomes with AMIO (▲). Non-irradiated solutions (controls): DOPG+DMPG alone (○) and DOPG+DMPG liposomes with AMIO (●). The line (□) represents the calculated curve obtained by subtracting the oxygen uptake of AMIO irradiated in DMPG liposomes from the oxygen uptake of irradiated AMIO–DOPG liposomes.

irradiation (45% after 30 min). It was similar to the total amount of oxygen consumed (5×10^{-7} mol) over the same period of time. This also supports the assumption that AMIO sensitized the oxidation of phospholipids.

DISCUSSION

Studies on interactions of AMIO with membranes have led to rather contradictory conclusions [21, 22, 25–29, 32]. The location of AMIO in membrane according to the predominance of hydrophobic or electrostatic interactions is as yet an open question. Previous quantitative X-ray diffraction studies have shown that, below the thermal phase transition and at a relatively low hydration of lipid, AMIO is located mainly within the hydrocarbon core of the membrane bilayer [22]. This is supported by the fluorescence data of Chatelain *et al.* [26] showing that the fluidifying effect of the drug below *Tt* and the rigidifying effect above this temperature were drug concentration dependent at values of *Ri* up to 0.33. This effect was not modified by the presence of an acidic phospholipid (4%). It was concluded that AMIO penetrated deeply into the lipid matrix, the hydrophobic nucleus being located along the lipid acyl chains (like cholesterol) and the amino residue at the lipid–water interface. The nature of the polar head group of the lipid bilayer was thought to be of secondary importance.

In contrast, Jendrasiak and Glisson [29] located AMIO near the phospholipid head group of the bilayer, on the basis of experiments using fully hydrated acidic phospholipids and a *Ri* of 0.6. More recently, these authors showed that AMIO modified the organization of bilayers in a pH-dependent manner. It was concluded that the interaction of the drug involves the head-group region rather than occurring deep in the hydrocarbon interior of the bilayer. In fact, preferential interaction of the cationic drug AMIO with acidic phospholipids is not contradictory with its insertion between the lipid molecules. Our results based on UV, light scattering and fluorescence polarization support this contention and provide further information on the interaction of AMIO with phospholipids. Regardless of the nature of the acyl chains (saturated or unsaturated), AMIO was observed to interact strongly with anionic lipids and also with neutral lipids but with a marked preference for the former, indicating that both hydrophobic and electrostatic forces are involved in these interactions. In particular, the marked change observed in the optical properties of AMIO–DMPG (or AMIO–DOPG) liposomes for a $Ri = 1$ was suggestive of an electrostatic interaction between the positively charged AMIO and the negatively charged PG molecules. Electrostatic interactions between AMIO and lipid may greatly facilitate the penetration and dispersion of the drug into the hydrophobic core of the lipid. This might explain why the effects of AMIO were observed for smaller *Ri* values with PG molecules than with PC molecules.

Our fluorescence polarization results lent support to this assumption. The effect of AMIO on the polarization rate of DPH was greater in the DMPG liposomes than in the DMPC liposomes, especially when the lipids were in the gel state. This may indicate that AMIO did not mix as well with DMPC as with DMPG under these conditions. In fact, electrostatic interactions could lead to high interfacial concentrations of the drug, while repulsions between drug molecules could favor a random distribution in the lipid matrix. This possibility has been suggested by Ferreira *et al.* [25] in an investigation of the modifications of liposomes induced by AMIO according to its ionization state. On the other hand, AMIO molecules which are less attracted by DMPC vesicles may remain partly in the bulk phase where they may be organized in clusters at these high concentrations (2×10^{-5} M), and so fail to destabilize lipids in the gel state. AMIO only penetrated between the neutral phospholipid molecules when the temperature was increased.

This explanation is in line with our size determinations of liposomes which have shown that the penetration and disruptive effects of AMIO were quantitatively greater in the DMPG liposomes and appeared earlier than in the DMPC liposomes. Such effects have been reported for other lipophilic drugs like Amphotericin B. Addition of Amphotericin B to liposomes has been shown to induce a characteristic disruption of the vesicles followed, at high drug concentration, by a complete loss of liposomal structures [33, 34].

As shown above, the location of the drug depends on many parameters such as the physical properties of the medium (pH, T^0, ionic strength), the nature and the hydration of the membrane model system used (liposomes, monolayers), the drug to lipid ratio and the proportion of acidic lipids in the liposomes. The whole set of our data indicates that the higher the concentration of acidic lipid in the liposome (in

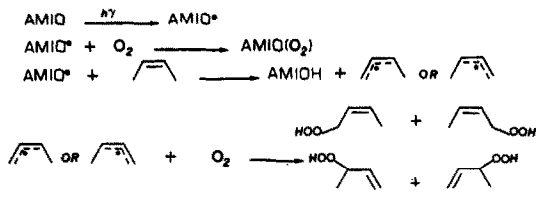

Fig. 6. Proposed mechanism for the AMIO-photosensitized peroxidation of lipids.

a domain or a leaflet of the membrane), the larger will be the interaction with AMIO. These interactions are facilitated with lipids in the liquid state and are likely to correspond to an insertion of the drug molecules between lipid molecules. At elevated *Ri*, the drug will thus tend to have a destructuring effect on liposome or membrane organization, especially in systems with a high acidic lipid content.

In the second part of this study, we examined the photosensitizing properties of AMIO, with the assumption that AMIO was incorporated in the DOPG liposomes. The oxygen uptake by DOPG under irradiation in the presence of AMIO was largely higher than that observed in the absence of drug. This indicated that a drug-photosensitized oxidation process occurred in the medium. The fact that oxygen uptake matched that expected for the oxidation of the missing unsaturated fatty acids (as estimated by gas chromatography) was a good argument in favor of the involvement of a peroxidation of the unsaturated phospholipids. This can be accounted for by a conventional mechanism, as it is known that AMIO is dehalogenated via homolytic cleavage of the carbon–halogen bond under irradiation [17, 18]. The radical deriving from the drug may abstract an allylic hydrogen from the lipid, yielding an easily oxidizable radical (Fig. 6). As can be seen from this mechanism, the proximity of AMIO with the lipid double bond is required for the peroxidation to occur. The penetration of AMIO in the hydrophobic core of the membrane appears as a key factor of the photosensitization process.

To conclude, this paper shows a potent interaction of AMIO with acidic phospholipids. The drug can insert between the lipid molecules to provoke destruction of the bilayer structure with increasing concentrations of the drug. It should be noted that the AMIO concentrations used in the present work (2×10^{-6}–4.3×10^{-4} M) were greater than those usually found in human therapeutics (1–2.5 μg, i.e. 1.7–4.3×10^{-6} M). However, there are many clinical reports in the literature which describe an accumulation of AMIO in organs (particularly skin, adipose tissue, liver and lung) after long-term therapy. The combination of drug insertion in the cellular membranes of the loaded tissues and drug photochemical reactivity which promotes lipid peroxidation might be partly responsible for the photohemolysis and phototoxic effects observed *in vitro* and *in vivo* after long-term therapy with AMIO.

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REFERENCES

1. Baudine A, Chaillet F, Charlier R and Hossler A, Recherche dans la série des benzofurannes XXVI. Effects pharmacologiques généraux de l'amiodarone. *Arch Int Pharmacodyn* **169**: 469–481, 1967.
2. Marcus FI, Fontaine GH, Franck R and Grosgageat Y, Clinical pharmacology and therapeutic applications of the antiarrhythmic agent, amiodarone. *Am Heart J* **101**: 480–493, 1981.
3. Singh BN, Nademance K, Ibeda N and Kannan R, Pharmacology and electrophysiology of amiodarone: experimental and clinical correlations. In: *New Aspects of the Medical Treatment of Tachyarrhythmias (Int. Symp.) 1982* (Eds. G. Briethardt and F. Loogen) pp. 46–56. Urban and Scharzenberg, Munich, 1983.
4. Gottlieb S and Sonnenblick M, Effect of amiodarone on blood lipids. *Am J Cardiol* **63**: 1540, 1989.
5. Kasim SE, Bagchi N, Brown TR, Khilnani S, Jackson K, Steinman RT and Lehmann MH, Amiodarone-induced changes in lipid metabolism. *Horm Metab Res* **22**: 385–388, 1990.
6. Singh BN and Koonlawee N, Amiodarone and thyroid function: clinical implications during antiarrhythmic therapy. *Am Heart J* **106**: 875–869, 1983.
7. Martin WJ, Kachel DL, Vilen T and Natarajan V, Mechanism of phospholipidosis in amiodarone pulmonary toxicity. *J Pharmacol Exp Ther* **251**: 272–278, 1989.
8. Kannan R, Sarma JSM, Guna G and Venkataraman K, Tissue drug accumulation and ultrastructural changes during amiodarone administration in rats. *Fundam Appl Toxicol* **13**: 793–803, 1989.
9. Canada AT, Lesko LJ, Haffajee CI, Johnson B and Asdourian GK, Amiodarone for tachyarrhythmias: pharmacology, kinetics, and efficacy. *Drug Intell Clin Pharm* **17**: 100–104, 1983.
10. Rappersberger K, Honnigsmann H, Ortel B, Tanew A, Konrad K and Wolff K, Photosensitivity and hyperpigmentation in amiodarone-treated patients: incidence, time course, and recovery. *J Invest Dermatol* **93**: 201–209, 1989.
11. Wanet J, Acthen G, Barchewitz G, Mestdagh C and Vastesaeger M, Amiodarone et dépôts cutanés. Etude clinique et histologique. *Ann Dermatol Syphiligr* **98**: 131–140, 1971.
12. Baret M, Les complications dermatologiques de l'amiodarone. Thése de la Faculté de Médecine de Brest, 1973.
13. Barchewitz G, Laurent G and Jamaer M, Surcharge tissulaire à pigment brun sous amiodarone. *Arch Int Pharmacol Ther* **184**: 59–67, 1970.
14. Geerts ML, Amiodarone pigmentation. An electron microscopic study. *Arch Belg Dermatol Syphiligr* **27**: 339–351, 1971.
15. Hassan T, Kochevar IE and Abdulah D, Amiodarone phototoxicity to human erythrocytes and lymphocytes. *Photochem Photobiol* **40**: 715–719, 1984.
16. Bose B, Agarwal S and Chatterjee SN, UV-A induced lipid peroxidation in liposomal membrane. *Radiat Environ Biophys* **28**: 59–65, 1989.
17. Paillous N and Verrier M, Photolysis of amiodarone, an antiarrhythmic drug. *Photochem Photobiol* **47**: 337–343, 1988.
18. Li AS and Chignell CF, Spectroscopic studies of cutaneous photosensitizing agents IX. A spin trapping

study of the photolysis of amiodarone and desethylamiodarone. *Photochem Photobiol* **45**: 191–197, 1987.
19. Riva E, Gerna M, Neyroz R, Urso R, Bartosek I and Guaitani A, Pharmacokinetics of amiodarone in rats. *Cardiovasc Pharm* **4**: 270–275, 1982.
20. Debbas MMG, Du Cailor C, Sassine A, Derancourt J, Demaille J and Puech P, Determination of cardiac and plasma drug levels during long-term amiodarone therapy. *Eur J Clin Invest* **13**: 123–127, 1983.
21. Chatelain P and Laruel R, Amiodarone partitioning with phospholipid bilayers and erythrocyte membranes. *J Pharm Sci* **74**: 783–784, 1985.
22. Trumbore M, Chester DW, Moring J, Rhodes D and Herbette LG, Structure and location of amiodarone in a membrane bilayer as determined by molecular mechanistics and quantitative X-ray diffraction. *Biophys J* **54**: 535–543, 1988.
23. Ravin LJ, Shami EG, Intoccia A, Rattie E and Joseph G, Effect of polysorbate 80 on the solubility and *in vivo* availability of 2-butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride. *J Pharm Sci* **58**: 1242–1245, 1969.
24. Ravin LJ, Shami EG and Rattie ES, Micelle formation and its relationship to solubility behavior of 2-butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride. *J Pharm Sci* **64**: 1830–1833, 1975.
25. Ferreira J, Brasseur R, Chatelain P and Ruysschaert JM, Properties of amiodarone monolayer spread at the air–water interface. *J Pharm Pharmacol* **38**: 561–566, 1986.
26. Chatelain P, Laruel R and Gillard M, Effect of amiodarone fluidity and Na^+/K^+ ATPase activity in rat-brain synaptic membranes. *Biochem Biophys Res Commun* **129**: 148–154, 1985.
27. Chatelain P, Ferreira J, Laruel R and Ruysschaert JM, Amiodarone induced modifications of phospholipid physical state. *Biochem Pharmacol* **35**: 3007–3013, 1986.
28. Ferreira J, Chatelain P, Caspers J and Ruysschaert JM, Ionisation state of amiodarone mediates its mode of interaction with lipid bilayer. *Biochem Pharmacol* **36**: 4245–4250, 1987.
29. Jendrasiak GL and Glisson G, A multinuclear NMR study of liposomes containing the antiarrhythmic drug, amiodarone. *Biophys J* **53**: 77a, 1988.
30. Huang C, Studies on phosphatidylcholine vesicles. Formation and physical characteristics. *Biochemistry* **8**: 344–352, 1969.
31. Bangham AD, Standish MM and Watkins JC, Diffusion of univalent ions across the lamellae of swollen phospholipids. *J Mol Biol* **13**: 238–252, 1965.
32. Jendrasiak GL, McIntosh TJ, Riebero A and Porter RS, Amiodarone–liposome interaction: a multinuclear NMR and X-ray diffraction study. *Biochim Biophys Acta* **1024**: 19–31, 1990.
33. Grant CWM, Hamilton KS, Hamilton KD and Barber KR, Physical biochemistry of a liposomal amphotericin B mixture used for patient treatment. *Biochim Biophys Acta* **984**: 11–20, 1989.
34. Janoff AS, Boni LT, Popescu MC, Minchey SR, Cullis PR, Madden TD, Tarashi T, Gruner SM, Shyamsunder E, Tate MW, Mendelsohn R and Bonner D, Unusual lipid structures selectively reduce the toxicity of amphotericin B. *Proc Natl Acad Sci USA* **85**: 6122–6126, 1988.