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Spin-labeled amphotericin B: synthesis, characterization, biological and spectroscopic properties

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A biologically active spin-labeled derivative of amphotericin B has been synthesized by the nucleophilic addition of amphotericin B to 4-(2-iodoacetamido)-2,2′,6,6′-tetramethylpiperidine-*N*-oxyl in dimethylsulphoxide at 40°C. The derivative is a moderately water-soluble compound which displays the same biological activity of the parental compound against the sensitive organism *Leishmania mexicana*; also, the rates of proton-cation exchange induced by the two compounds in large unilamellar liposomes are indistinguishable. The ESR spectra of spin-labeled amphotericin B in lipid vesicles indicate a high degree of motion, very similar to that encountered for the compound in aqueous solutions at neutral pH and in deoxycholate micelles, and suggest that the structures formed by the antibiotic in membranes are composed by a small number of molecules. In contrast, the spectra of the labeled antibiotic in ethanol, diethyl ether and dimethylformamide indicate restricted motion and exchange interactions, probably resulting from the micellar aggregation induced in these media. Ascorbate at 10 mM is able to reduce completely the nitroxide group of the labeled antibiotic in lipid vesicles in less than 30 s, indicating that an asymmetric disposition of the antibiotic molecules across the membrane is capable of inducing its biological and ionophoric properties. Ni^{2+} and Cu^{2+} produce moderate exchange broadening of the ESR signal of spin-labeled amphotericin B in lipid vesicles; the comparison of this phenomenom with the exchange broadening produced by the same ions in the ESR spectrum of 2,2′,6,6′-tetramethylpiperidine-*N*-oxyl in water solution suggests an specific Cu^{2+}–amphotericin B interaction in membranes.

Introduction

The polyene antibiotics constitute an important group of macrocyclic lactonic compounds whose biological action is mediated by an alteration of the membrane permeability of sensitive organisms, which are those that incorporate sterols in their plasmatic membranes [22,23,32]. Amphotericin B, an heptaene with a mycosamine residue linked to the hydroxyl at C-19 of the macrolide ring, is the most frequently used antibiotic in the treatment of systemic fungal infections; it is also the best characterized, including its crystal and molecular structure [19,.33], binding to sterols [8,35,36,44,47] and biological specificity [26,27,32].

The most widely accepted model of the molecu-

Abbreviations: ESR, electron spin resonance; TLC, thin-layer chromatography; LUV, large unilamellar lipid vesicles; SUV, sonicated unilamellar lipid vesicles; TEMPO, 2,2′,6,6′-tetramethylpiperidine-*N*-oxyl.

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lar entities created by amphotericin B in membranes is derived from studies on the effect of this and related compounds on the permeability of black lipid membranes to ions, water and small non-electrolytes, when the antibiotic is added to both sides of the membrane; under these conditions the permeability data are consistent with the formation of aqueous pores of approximately 8 Å in diameter [17]. The pore is pictured as being formed by the aposition of two 'half-pores' situated at each side of the membrane, each of them formed by a circular arrangement of eight amphotericin B molecules, with their hydroxyl groups oriented towards the center of the pore, intercalated with eight β-OH sterol molecules [13,17]. However, the understanding of the biological activity of these compounds requires the study of the permeability effects and the structures formed by the unilateral addition of the antibiotic. The addition of amphotericin B and related compounds to only one side of black lipid membranes and liposomes elicits increments in the ionic permeability of these structures at concentrations which are comparable to those active against cells and tissues but considerably greater than those required by two-sided adddition or by the inclusion of the antibiotic in the lipid phase previously to the membrane formation [30,46]. Also, the conductance induced by one-sided addition of the antibiotic is cationic, in contrast with the anionic conductivity observed by two-sided addition [30]. In vivo, the addition of amphotericin B to toad bladder increased the permeability to small solutes such as KCl and thiourea but the water flux under an osmotic gradient was unaffected [28]; Rich et al. [39] failed to demonstrate any effect of the antibiotic on the osmotic water permeability of the erythrocyte. Finally, recent studies on the permeability changes induced by one-sided addition of amphotericin B to large unilamellar lipid vesicles and *Leishmania mexicana* membranes, using an osmotic method, revealed that under these conditions the antibiotic produced specific ionic permeability, not affecting the osmotic behaviour of these membranous structures [9–11]. All these facts indicate that, under conditions resembling those under which the antibiotic is active in vivo, the structures formed by it in membranes are not simple aqueous pores but behave as specific ionic channels, a fact incompatible with the classical pore model and its refinements [13,17,46]. Single ionic channels formed by one-sided or two-sided addition of low ($\sim 10^{-8}$ M) concentrations of amphotericin B to black lipid membranes have been reported and characterized [6,14,15].

Despite this controversy, very little direct information has been gathered on the molecular organization of the structures formed by amphotericin B in membranes. Proton magnetic resonance [20] and electron spin resonance (ESR) of spin-labeled fatty acids and cholestane probes [1,18,36,37] only report the perturbation of the lipid matrix of the membrane by high (comparable to the sterol) concentrations of the antibiotic, but do not give details of the structures formed by it. Circular dichroism studies [2,3,47] reveal the presence of several spectroscopic species of amphotericin B formed in membranes under different conditions, some of them correlated with the permeability changes observed, but no molecular conformation or aggregate has been asigned to any given spectroscopic species. In this paper we report the synthesis and the biological and spectroscopic characterization of a biologically active spin-labeled derivative of amphotericin B; we show by ESR that the active structures formed by it in membranes are small aggregates that are disposed asymmetrically across the lipid bilayer and we present evidence for an specific Cu^{2+}–amphotericin B interaction in membranes.

Materials and Methods

Amphotericin B (Sigma Chemical Company) was purified by precipitation and washing in diethyl ether, followed by partition chromatography on lipophilic Sephadex LH-20, using as eluent chloroform/methanol/water (20 : 10 : 1, v/v; Ref. 16). The synthesis of the spin-labeled derivative was carried out essentially as described by Falkowski et al. [16] for the preparation of the *N*-glycosyl derivatives; briefly, purified Amphotericin B was dissolved (30 mM) in dimethylsulphoxide and incubated with a 0.5 molar excess of 4-(2-iodoacetamido)-2,2′,6,6′-tetramethylpiperidine-*N*-oxyl (Aldrich Chemical Company) with stirring at 40°C in the dark. The reaction

was monitored by thin-layer chromatography (TLC) on silica gel plates, using as solvent ethyl acetate/acetic acid/water (4:1:1, v/v); it was judged as completed in 72 h. *Leishmania mexicana* (stock NR) promastigotes were cultured in a modified liver infusion-tryptose medium, supplemented with 10% foetal calf serum, at 26°C [4]. Cell density was determined using an electronic particle counter (Coulter, model ZBI); for the determination of the antibiotic effect of amphotericin B and its spin labeled derivative, the compounds were added as dimethylformamide solutions (final dimethylformamide concentration < 0.1%), to an initial cell density of $1 \cdot 10^7$ and growth was monitored every 12 h for 72 h. Egg phosphatidylcholine/ergosterol (84:16, mol/mol) large unilamellar lipid vesicles (LUV) were prepared using the reverse-phase method of Szoka and Papahadjoupoulos [43]; liposomes formed by this procedure are predominantly unilamellar, with a diameter between 0.05 and 0.2 μm, as shown by negative staining electron microscopy [10]. Sonicated lipid vesicles (SUV) were produced by subjecting LUV to prolonged (60 min) ultrasonic irradiation using a bath sonicator (Laboratory Supplies Company, Inc.); the formation of an essentially homogeneous population of small unilamellar vesicles, about 22 nm in diameter, was verified by electron microscopy as indicated above [10,24]. The sonicated vesicles retain the proton impermeability characteristic of LUV (see below). Cation-proton exchange induced by the polyene antibiotics was measured at 25°C by a modification of the procedure described by Deamer and Nichols [12]: LUV were prepared in 40 mM aspartic acid, 250 mM K_2SO_4 (pH 4), diluted to 3 mM lipids in the same buffer and the pH of the external solution adjusted to 7 with KOH. After obtaining a stable external pH, appropriate amounts of a stock solution of amphotericin B or the spin-labeled derivative (1.5 mM in dimethylformamide) were added and the cation-proton exchange was determined recording continuously the acidification of the external solution, using a Radiometer GK2321C electrode connected to a PHM-84 potentiometer, coupled to Sargent-Welch XKR plotter. Total exchangeable protons were determined in an identical sample by adding Triton X-100 (final concentration, 0.2%, v/v). The concentration of the stock solutions of the antibiotics were adjusted using a molar absorption coefficient in dimethylformamide at 409 nm of $1.23 \cdot 10^5$ for Amphotericin B [38] and $5.3 \cdot 10^4$ for the spin-labeled derivative (see Results). UV-VIS spectra were obtained in a Varian-Techtron Super Scan 3 recording spectrophotometer. X-band ESR spectra were obtained in a Varian E-104 spectrometer; microwave power and modulation (100 kHz) amplitudes were adjusted for optimal signal-to-noise ratios, without spectral distortion. All biochemical reagents were of the maximal purity available and were purchased from Sigma Chemical Company. Other reagents were of analytical reagent grade and were obtained from Merck, A.G. Sephadex LH-20 is a product of Pharmacia Fine Chemicals.

Results

The reaction of amphotericin B with 4-(2-iodoacetamido)-2,2′,6,6′-tetramethylpiperidine-*N*-oxyl, most probably mediated by the nucleophilic attack of the single amino group of the antibiotic to the electrophilic carbonyl of the iodoacetamide, produced a compound which gave a symmetric spot by TLC ($R_F = 0.49$, compared with 0.43 for the parental compound); the yield was 75%. The derivative can be partly solubilized in water by titrating to neutral pH. The minimal growth in-

TABLE I

CATION-PROTON EXCHANGE INDUCED BY AMPHOTERICIN B AND ITS SPIN-LABELED DERIVATIVE ACROSS LARGE UNILAMELLAR LIPID VESICLES

The cation-proton exchange induced by the polyenes in LUV was measured at 25°C as described in Materials and Methods. Δ(pH) is the total pH change observed in the presence of Triton X-100 (0.2% v/v). Results are means ± standard deviation of a triplicated experiment.

Condition	$(dpH/dt)/\Delta(pH)$ (min^{-1})	%
Amphotericin B (3 μM)	$(27 \pm 4) \cdot 10^{-3}$	100.0
Amphotericin B (3 μM) + tetraethylammonium (10 mM)	$(12 \pm 1) \cdot 10^{-3}$	45.3
Spin-labeled amphotericin B (3 μM)	$(34 \pm 4) \cdot 10^{-3}$	100.0
Spin-labeled amphotericin B (3 μM) + tetraethylammonium (10 mM)	$(16 \pm 1) \cdot 10^{-3}$	47.9

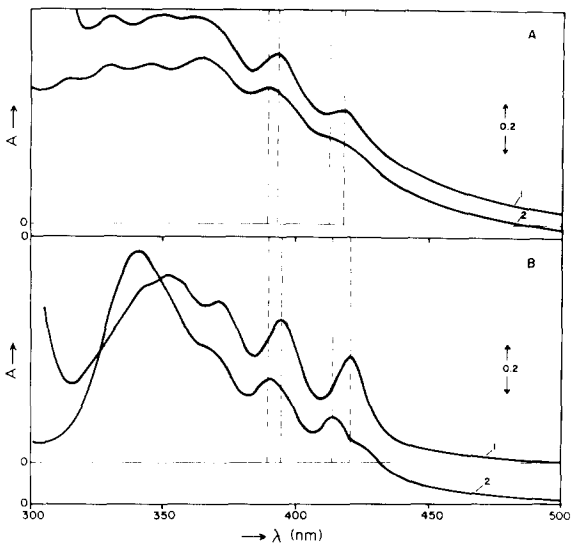

Fig. 1. Electronic spectra of spin-labeled amphotericin B (A) and amphotericin B (B) in the presence of SUV (5 mM lipids, spectrum 1) and in water suspension (spectrum 2). Concentration of the antibiotics: 10 μM. Other details are described in Materials and Methods.

hibitory concentrations and minimal lytic concentrations of amphotericin B and the spin-labeled derivative against promastigotes of *Leishmania mexicana* are identical (0.4 μM and 0.8 μM, respectively, under the conditions described in Materials and Methods). The cation-proton exchange induced by both compounds across LUV are also indistinguishable (Table I), as are the inhibitory effects of tetraethylammonium on this phenomenon (Table I; see Ref. 5). The electronic spectra of amphotericin B and the spin-labeled derivative in water and in the presence of SUV are presented in Fig. 1: it can be seen that in both cases there is a marked sharpening and red shift of the whole spectrum in the presence of the lipid vesicles. The spectrum of the derivative in dimethylformamide (not shown) is broadened and distorted and the molar absorption coefficient is significantly smaller when compared with that of the parental compound (see Materials and Methods).

The X-band ESR spectra of spin-labeled amphotericin B in a series of solvents and in different aqueous suspensions are presented in Fig. 2. Most noticeable is the strong similarity of

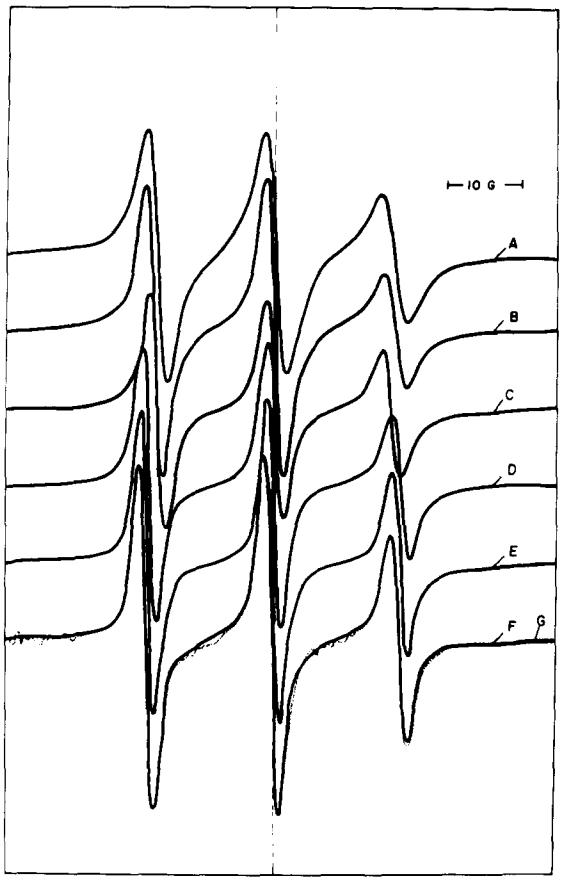

Fig. 2. X-band ESR spectra of spin-labeled amphotericin B (80 μM, A to F) in: (A) ethanol; (B) diethyl ether; (C) dimethylformamide; (D) 1% (w/v) deoxycholate in water; (E) water (pH 7); (F) SUV (20 mM lipids, 8 molecules per vesicle); (G, dotted line) 20 μM spin-labeled amphotericin B in SUV (20 mM lipids, 2 molecules/vesicle). Other details are described in Materials and Methods.

the spectra of the compound in aqueous solution, in deoxycholate micelles (0.05 antibiotic molecules/micelle, assuming a micellar aggregation number of 14; Ref. 25) and in SUV (2 and 8 molecules per vesicle); all of them reflect high mobility of the nitroxide group, although much smaller than that of a free 2,2′,6,6′-tetramethylpiperidine-*N*-oxyl (TEMPO) molecule in water (Table II). The spectrum in dimethylformamide is more broadened and in diethyl ether and ethanol sereve broadening and distortion is observed. Table II gives an approximate quantitative analysis of the spectra presented in Fig. 2; it can be seen

TABLE II

HYPERFINE SPLITTINGS AND MOBILITY OF SPIN-LABELED AMPHOTERICIN B IN DIFFERENT MEDIA

Correlation times (t_c), calculated assuming isotropic reorientation and Lorenzian lineshapes [40,42] and 'empirical motion parameters' [34] were obtained from the spectra shown in Fig. 2. Other details are described in Materials and Methods.

Condition	A_o (gauss)	R_i	t_c (ns)
Spin-labeled amphotericin B in:			
ethanol	16.00	3.08	0.70
diethyl ether	16.25	2.35	0.74
dimethylformamide	15.75	2.16	0.46
deoxycholate micelles (1% (w/v) in water)	16.90	1.51	0.42
SUV (2 and 8 molecules/vesicle)	16.80	1.40	0.35
water (pH 7)	16.85	1.39	0.34
TEMPO in water	17.15	0.07	0.015

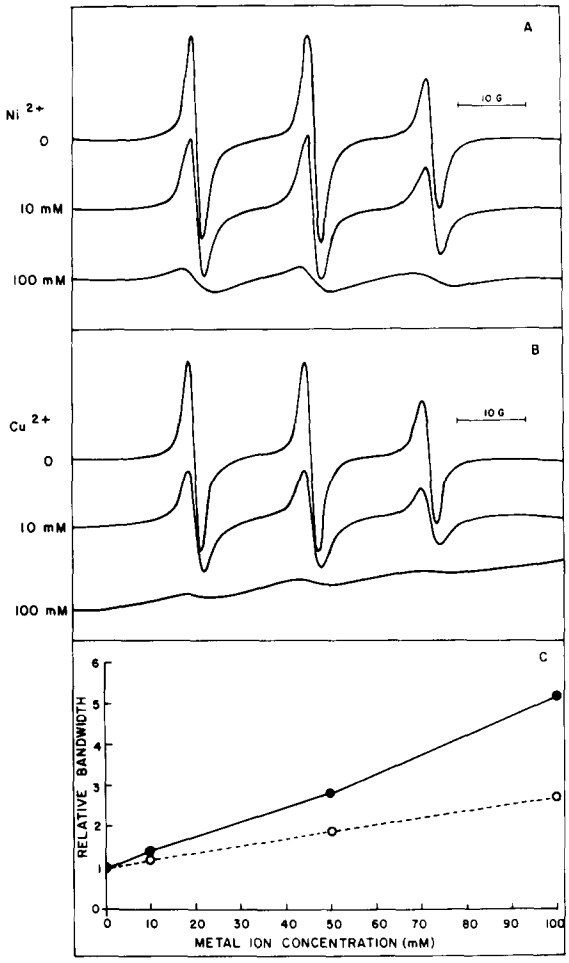

Fig. 3. Exchange relaxation induced by Ni^{2+} and Cu^{2+} on spin-labeled amphotericin B in lipid vesicles. Top panel: X-band ESR spectra of spin-labeled amphotericin B in SUV (8 molecules per vesicle) in the presence of the indicated concentrations of Ni^{2+}. Middle panel: same as above but in the presence of the indicated concentrations of Cu^{2+}. Bottom panel: relative broadening as a function of the divalent ion concentration for Cu^{2+} (●) and Ni^{2+} (○). Other details are described in Materials and Methods.

that the hyperfine splittings reflect the solvent's polarity, indicating that the nitroxide group is directly exposed to it. The mobility of the nitroxide group, indicated by the correlation time [40,42] or the 'empirical motion parameter' (R_i; Ref. 34) is almost identical in water (pH 7) and SUV and very close to the value in deoxycholate micelles but decreases significantly in dimethylformamide, diethyl ether and ethanol; moreover, in the last two solvents the correlation between the two motional parameters derived from the spectra is lost. Ascorbate (10 mM) eliminates instantly (< 30 s) the ESR spectrum of spin-labeled amphotericin B, both in LUV and SUV, presumably as a consequence of the reduction of the nitroxide group.

Fig. 3 presents the exchange broadening (in the motional range of the polyene-bound nitroxide, Table II, the dipolar relaxation effects are negligible; see Refs. 29 and 41) produced by Ni^{2+} and Cu^{2+} on the ESR spectrum of spin-labeled amphotericin B in SUV (8 molecules per vesicle; identical results were obtained with 2 molecules per vesicle); it shows that Cu^{2+} has a exchange relaxation constant which is twice that observed for Ni^{2+}. The study of the exchange broadening produced by these ions on the ESR spectrum of TEMPO in aqueous solution (data not shown; see Ref. 29) demonstrates that in the case of Ni^{2+} the exchange relaxation of the nitroxide in solution is 4-fold greater than that observed for spin-labeled amphotericin B in SUV; however, for Cu^{2+} the ratio of the two exchange relaxation constants is only 2.6.

Discussion

This is the first report, to the best of our knowledge, on the synthesis and characterization of a spin-labeled derivative of a polyene antibiotic. The spin-labeled compound displays the same biological and ionophoric properties of amphotericin B, as has been reported for the *N*-glycosyl derivatives [16]. Binding of the derivative to lipid vesicles, comparable to that of amphotericin B, was evident from the alteration of the electronic spectra of these compounds produced by the presence of the lipid, as previously reported [35,38]. The restricted motion of the nitroxide group, when compared with that of TEMPO in solution (Table II), confirms its binding to a macromolecular structure. Furthermore, the correlation of the hyperfine splittings with the solvent's polarity [21] is consistent with the attachment of this group to the polar mycosamine moiety of the antibiotic and the expected exposition of this region of the molecule to the solvent. The very similar mobility of the nitroxide group of the labeled antibiotic in neutral aqueous solutions, in deoxycholate micelles (one antibiotic molecule for every 20 micelles) and in SUV, together with the lack of noticeable exchange broadening effects in these spectra (indicated by the exact correlation of the two motional parameters calculated from them), clearly indicate that the active structures formed by the antibiotic in lipid vesicles are highly mobile and composed of a small number of molecules. Consistent with this interpretation are the values of Hill coefficients ($n = 2$–3) for the activation of KNO_3 and urea permeability in LUV by amphotericin B [9,10] and the data of Bolard et al. [3] which indicate that two amphotericin B molecules per sonicated lipid vesicle are enough to release the full titratable proton's content of the vesicle. Aggregation of pairs of bile salts' molecules, which are structurally analogous to polyene antibiotics, have been proposed by Mazer et al. [31] for 'mixed disk' phospholipid-bile salts micelles. The very fast and complete reduction by ascorbate of the nitroxide of the labeled antibiotic in both SUV and LUV confirms the exposition of the group to the solvent and proves that a completely asymmetric disposition of the antibiotic molecules in the membrane is sufficient to elicit its biological and ionophoretic properties [46]. On the other hand, the broadened and distorded spectra of the antibiotic in diethyl ether and ethanol suggest restricted and anisotropic motion of the nitroxide groups and/or exchange and dipolar interactions between them, probably resulting from the micellar aggregation of the antibiotic induced by these solvents [22,45]. Finally, both the ESR and electronic spectra of the derivative in dimethylformamide, a remarkably good solvent of amphotericin B, indicate that in this medium the spin-labeled antibiotic is not in true solution and moderately-sized aggregates might be present.

The smaller Ni^{2+}- or Cu^{2+}-induced exchange relaxation of the nitroxide group of spin-labeled amphoreticin B in SUV compared with aqueous TEMPO solutions is consistent with the restricted mobility and accessibility of the macromolecule-bound group. However, the higher efficiency of the Cu^{2+}-induced exchange relaxation of the antibiotic bound group suggests a specific Cu^{2+}–spin-labeled amphotericin B interaction, which would be reflected in increased lifetimes of the collisional complexes [29,41]. Cheron et al. [7] have shown that Cu^{2+} drastically diminishes the amphotericin B-induced lysis of human erythrocytes, but does not affect the K^+ leakage produced by lower concentrations of the antibiotic; the authors suggest that the origin of this phenomenon is an interaction of Cu^{2+} with the erythrocyte membrane as they could not detect a direct Cu^{2+}–Amphotericin B interaction by Cu^{2+} ESR or amphotericin B circular dichroism. Our results indicate that there is an specific interaction between Cu^{2+} and the spin-labeled amphotericin B, which is probably related to the precipitation of the *N*-glucosyl derivative by this divalent cation, and could explain the stochiometric effect of Cu^{2+} on the amphotericin B-induced lysis [7].

Spin-labeled derivatives of polyene antibiotics, such as the one described here, should prove very useful in the unraveling of the molecular basis of their action.

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