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Amphotericin B Protects *cis*-Parinaric Acid against Peroxyl Radical-Induced Oxidation: Amphotericin B as an Antioxidant

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The antifungal effects of amphotericin B are believed to be due to two possibly interrelated mechanisms: an increase in permeation by binding to sterols in cellular membranes and a prooxidant effect causing oxidative damage in target cells. However, the seven conjugated double bonds in amphotericin B raise the possibility that it could be highly susceptible to autoxidation, causing an antioxidant effect. In the present study, we investigated the prooxidant and antioxidant properties of amphotericin B in a model system in which oxidation of a reporter molecule, *cis*-parinaric acid, was induced by azo initiators of peroxyl radicals. Since interactions of amphotericin B with sterols are essential for its pharmacological and toxic actions, we also studied the effects of cholesterol on the prooxidant and antioxidant properties of amphotericin B. Amphotericin B caused a noncollisional quenching of a characteristic fluorescence of cholesteryl *cis*-parinarate integrated in liposomes, suggesting the formation of amphotericin B–cholesteryl *cis*-parinarate complex. This effect of amphotericin B was ablated by increasing concentrations of cholesterol. We found that amphotericin B inhibited oxidation of *cis*-parinaric acid complexed with human serum albumin [using a water-soluble azo initiator, 2,2′-azobis(2-aminopropane)dihydrochloride] and in liposomes [using a lipid-soluble azo initiator, 2,2′-azobis(2,4-dimethylvaleronitrile)]. The inhibitory effect of amphotericin B on 2,2′-azobis(2,4-dimethylvaleronitrile)-induced peroxidation of *cis*-parinaric acid in liposomes was also diminished by cholesterol. The antioxidant effect of amphotericin B in this model system suggests that amphotericin B does not exert its pharmacological and toxicological responses through a prooxidant effect to cause damage in target cells.

Amphotericin B (AmB), a heptaene macrolide antibiotic, is widely used to treat systemic fungal diseases. Even after 30 years of clinical use and with the emergence of the newer drugs, AmB remains the drug of choice for the treatment of severe systemic infections. It is believed that AmB exerts its antifungal effects by two possibly interrelated mechanisms: (i) an increase in permeation by binding to sterols in cellular membranes (1, 3) and (ii) prooxidant effects causing oxidative stress (3), possibly accompanied by the formation of free radical intermediates (8). Alternatively, the presence of seven conjugated double bonds in AmB could result in it being susceptible to autoxidation, causing an antioxidant effect similar to those of carotenoids and retinoids. The cytotoxic properties of AmB can damage mammalian cells and can cause a variety of adverse effects including nephrotoxicity and anemia (2, 4, 17). Hence, understanding of the prooxidant and antioxidant properties of AmB could be of value in developing strategies that may be useful for improving antifungal therapy and minimizing the untoward effects of AmB. Support for this approach is that antioxidants protect erythrocytes from AmB-induced lysis (2). This raises the possibility that antioxidants and/or antioxidant enzymes can indeed be used to protect against AmB-induced side effects. However, the exact mechanism(s) involved in the autoxidation and potential oxidative effects of AmB has not been clearly elucidated.

Recently, it was demonstrated that a fluorescent natural polyunsaturated fatty acid, *cis*-parinaric acid (PnA; 9-*cis*, 11-*trans*, 13-*trans*, 15-*cis*-octadecatetraenoic acid), can be used as a sensitive and reliable reporting molecule for assaying lipid peroxidation in membranes and model systems (7). Four conjugated double bonds in PnA make it slightly more sensitive to free radical attack than most of the polyunsaturated fatty acid residues of membrane phospholipids (21). Due to this, PnA has been extensively used for studies of the antioxidant and prooxidant activities of different compounds including carotenoids and retinoids (20).

In the present study, we used PnA to investigate the prooxidant and antioxidant properties of AmB in a model system in which oxidation was induced by peroxyl radicals, the major source of free radicals causing membrane damage (10). Since interactions of AmB with sterols are essential for its pharmacological and toxic actions, we also studied the effects of cholesterol on the oxidation of AmB and PnA.

MATERIALS AND METHODS

Reagents. DL-α-Dipalmitoyl phosphatidylcholine (DPC), L-α-phosphatidic acid from egg phosphatidylcholine (PA), cholesterol (5-α-cholesten-3β-ol), deferoxamine mesylate, AmB, dimethyl sulfoxide (DMSO), and human serum albumin (hSA) were purchased from Sigma Chemical Co. (St. Louis, Mo.). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) and 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), which were used as lipophilic and hydrophilic radical initiators, respectively, were obtained from Polysciences, Inc. (Warrington, Pa.), and Wako Chemicals USA, Inc. (Richmond, Va.), respectively. PnA and cholesteryl *cis*-parinarate (Ch-PnA) were purchased from Molecular Probes (Eugene, Oreg.). The PnA-hSA complex was prepared by adding PnA (500 μg, 1.8 μmol) in 25 μl of DMSO to hSA (50 mg) in 1 ml of phosphate-buffered normal saline.

Preparation of liposomes. Lipid mixtures of DPC and PA (80 and 20 mol%, respectively) and a mixture of DPC, PA, and cholesterol (60, 20, and 20 mol%, respectively) dissolved in chloroform were dried under nitrogen. The dried lipid film was swollen in 0.1 M phosphate buffer (pH 7.4) at room temperature for 60 min under nitrogen, and then the mixture was vortexed. The lipid dispersion (4 mg of total lipids per 1 ml) was sonicated with an ultrasonic disintegrator (Cole Parmer Instrument Co., Chicago, Ill.) by using the microtip for 1 to 2 min to clarify the mixture and to form liposomes. AmB and PnA were incorporated into

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liposomes by addition of aliquots of their DMSO stock solutions to the liposome suspension to get lipid/AmB and lipid/PnA ratios of 50 to 170.

In preliminary experiments, we evaluated binding of amphotericin B to liposomes using size exclusion chromatography on Sephadex G-25. AmB was applied to the column (1.0 by 20.0 cm) as a 10 μM solution in phosphate buffer (0.1 M, pH 7.4, 1 ml) or as a mixture with liposomes (10 μM AmB, 0.5 mM phospholipids) in phosphate buffer (0.1 M, pH 7.4, 1 ml). In the latter case, AmB was added to preformed liposomes as a solution in DMSO (3 mM), and the mixture was preincubated for 5 min at 37°C. We found that 30% of the total amount of amphotericin B was recovered as high-molecular-weight aggregates, while 70% AmB eluted as low-molecular-weight aggregates and the monomeric form in the absence of liposomes. In the presence of liposomes, however, AmB coeluted exclusively with liposomes. We did not find any significant difference between binding of AmB to cholesterol-free liposomes or to cholesterol-loaded liposomes (20 mol% cholesterol).

Fluorescence measurements of PnA and Ch-PnA. Measurements of PnA and Ch-PnA fluorescence were carried out at 37°C in a thermostated cell holder of the RF-5301PC fluorescence spectrophotometer (Shimadzu Instruments Inc., Kyoto, Japan) at a scan speed of 1,800 nm/min. The excitation and emission wavelengths were 303 or 326 nm (slit width, 1.5 or 3 nm) and 420 or 433 nm (slit width, 5 nm), respectively.

HPLC assay of PnA. The samples were separated by isocratic reversed-phase high-pressure liquid chromatography (HPLC) by using a 5-μm Hewlett-Packard octyldecyl silane column (4.6 by 200 mm). The mobile phase was 0.05% acetic acid, 4.45% H_2O, and 95% methanol (flow rate, 1 ml/min). An HPLC system (model LC600; Shimadzu) equipped with a UV-visible light (VIS) photodiode array detector (model SPD M10A) was used. The detector output signal was processed and stored in digital form by using Shimadzu EZChrom software.

AAPH-induced oxidation of AmB. Oxidation of AmB (20 μM) was carried out in 0.1 M phosphate buffer (pH 7.4) at 37°C in the presence of hSA (0.25 mg/ml) or PnA-hSA complex (20 mM PnA, 0.25 mg of hSA per ml). In preliminary experiments, we studied the solubility of AmB in 0.1 M phosphate buffer (pH 7.4 at 37°C) by recording its optical spectra in the presence and in the absence of hSA (0.25 mg). A total of 1 to 20 μl of AmB dissolved in DMSO (3 mM) was added to the phosphate buffer. We found that the optical densities (at 386 and 409 nm) of AmB in the phosphate buffer were linearly dependent on its concentration within the range from 3 to 30 μM. At higher concentrations, the increase in the optical density was nonlinear. AmB's spectra were not affected by hSA at a concentration of 0.25 mg/ml. On the basis of these results, we used 20 μM AmB in our experiments on AAPH-induced oxidation. The reaction was induced by adding of 50 mM AAPH to the reaction mixture. The oxidation of AmB was monitored with a Shimadzu 160U UV-VIS spectrophotometer at 420 nm.

AAPH-induced oxidation of PnA-hSA complex. Oxidation of PnA (20 μM) in the PnA-hSA complex was carried out in 0.1 M phosphate buffer at 37°C. The reaction was induced by adding AAPH (50 mM) to the reaction mixture. The oxidation of PnA was monitored with a Shimadzu RF-5301PC fluorescence spectrophotometer as described above. The measurements of fluorescence were performed every 5 min for 60 min.

AMVN-induced oxidation of PnA in liposomes. Cholesterol-free or cholesterol-loaded liposomes into which PnA was incorporated were incubated in 0.1 M phosphate buffer at 37°C in the presence of 1 mM AMVN. The final concentrations of lipids and PnA were 0.5 mM and 3 μM, respectively. Deferoxamine mesylate (100 μM) was added to prevent uncontrolled oxidation by adventitious iron. The reaction was started by increasing the sample temperature to 37°C immediately after the addition of AMVN (1.0 mM, zero time). The measurements of fluorescence of PnA were performed every 5 min for 60 min as described above.

Quenching of Ch-PnA fluorescence by AmB. Liposomes containing Ch-PnA and different concentrations of cholesterol (0, 5, 10, and 20 mol% or 0, 0.025, 0.05, and 0.1 mM, respectively) were prepared. AmB at the required concentration (0, 0.06, 0.15, 0.3, 0.6, 1.5, 3.0, and 6.0 μM) was integrated into the liposomes. The fluorescence of Ch-PnA was measured by using an RF-5301PC fluorescence spectrophotometer as described above.

Statistics. Data are presented as means ± standard errors of the means (SEMs). Changes in variables for the different incubations were analyzed by analysis of variance and covariance for repeated measures. If changes between groups were significant, unpaired Student's *t* test was performed for data from every time point. Differences among means were considered significant when the value was $P < 0.05$.

RESULTS

AAPH-induced oxidation of AmB and PnA. Initially, we performed experiments with a water-soluble complex of PnA with human serum albumin (PnA-hSA). The oxidation of PnA was initiated with a water-soluble generator of peroxyl radicals, AAPH. Figure 1 presents the fluorescence emission and excitation spectra of PnA-hSA during the oxidation. PnA oxidation was directly monitored by measuring the changes in its fluorescence intensity at 420 nm (Fig. 1). Incubation of PnA-hSA with AAPH for 60 min at 37°C induced oxidation of 55% PnA. In the presence of 20 μM AmB (PnA/AmB ratio, 1:1), the PnA oxidation was significantly decreased ($P < 0.01$) by 20% for 60 min (Fig. 2). The time to achieve a 40% reduction in fluorescence intensity increased from 38 ± 1 min to 46 ± 2 and 54 ± 4 min with 10 and 20 μM AmB, respectively.

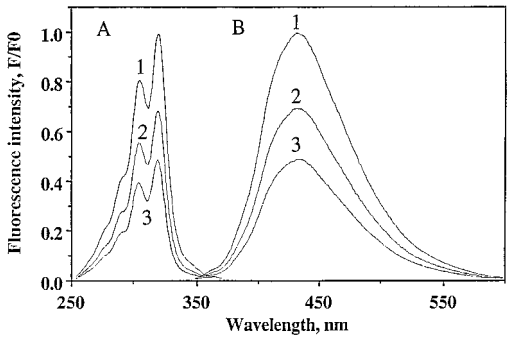

FIG. 1. Excitation (A) and emission (B) fluorescence spectra of PnA-hSA in the absence (curve 1) and in the presence (curves 2 and 3) of AAPH. The reaction mixture contained 0.1 M phosphate buffer (pH 7.4 at 37°C), 100 mM deferoxamine mesylate, PnA-hSA complex (20 mM PnA, 0.25 mg of hSA per ml), and 50 mM AAPH. (A) Excitation spectra were recorded by using an emission wavelengths of 420 nm (slit width, 5 nm); (B) emission spectra were recorded by using an excitation wavelength of 326 nm (slit width, 3 nm). Times of incubation were as follows: curve 1, 0 min, 2, 30 min; 3, 60 min. No appreciable changes in fluorescence were observed during incubation without AAPH.

In separate experiments, we studied the AAPH-induced oxidation of 20 μmol of AmB by measuring its characteristic absorbance. AAPH oxidized 16 μmol of AmB during 60 min of incubation at 37°C (Fig. 3). When these measurements were performed in the presence of PnA, only 11 μmol of AmB was oxidized during the same period of time (Fig. 3). These observations indicate that PnA and AmB mutually inhibit the oxidation of each other, most likely by competing for the AAPH-derived peroxyl radicals.

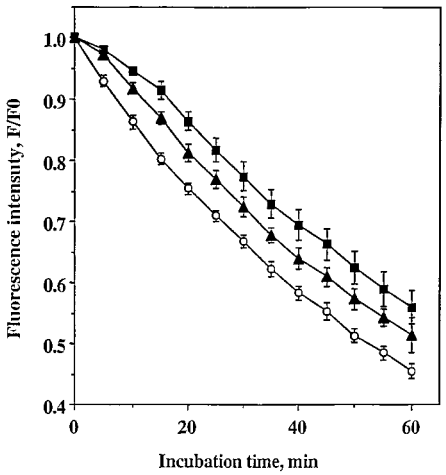

FIG. 2. Time course of AAPH-induced oxidation of PnA in PnA-hSA complex detected by fluorescence decay. The reaction mixture contained 0.1 M phosphate buffer (pH 7.4 at 37°C), 100 mM deferoxamine mesylate, PnA-hSA complex (20 mM PnA, 0.25 mg of hSA per ml), and 50 mM AAPH. Open circles, control; Solid triangles, plus 10 mM AmB; solid squares, plus 20 μM AmB. The results are means ± SEMs ($n = 4$). The statistical analysis was performed for each time point (10 to 60 min) and yielded significant differences between the control curve and both AmB curves, with $P < 0.05$.

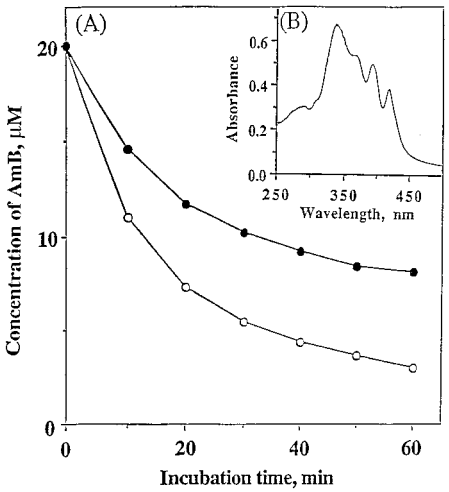

FIG. 3. Time course of AAPH-induced oxidation of AmB detected spectrophotometrically (A) and the UV-VIS spectrum of AmB (B). (A) The reaction mixture contained 0.1 M phosphate buffer (pH 7.4 at 37°C), 100 mM deferoxamine mesylate, 20 mM AmB, and 50 mM AAPH. Control, 0.25 mg of hSA per ml (open circles); in addition to the reaction mixture used in control experiments, PnA-hSA complex was added (20 mM PnA, 0.25 mg/ml hSA) (solid circles). (B) The reaction mixture contained 0.1 M phosphate buffer (pH 7.4 at 37°C) and 20 μM AmB.

In addition to its free form, in vivo AmB is likely to form complexes with cholesterol and cholesterol esters in cell membranes or lipoproteins (1). In membranes competition of AmB and PnA for peroxyl radicals and interactions of the radicals formed from either PnA or AmB may be significantly different due to restrictions imposed by the hydrophobic membrane environment. Thus, in subsequent experiments, we used a model system in which both PnA and AmB were integrated into liposomes (7, 20).

Quenching of Ch-PnA fluorescence by AmB. In preliminary experiments, we proportioned the concentration of phospholipids in liposomes, PnA, and AmB to prevent possible artifacts in fluorescence measurements due to nonspecific effects (e.g., fluorescence self-quenching by high concentrations of PnA) and to convert all AmB in the incubation medium to the membrane-bound state. The formation of the AmB-cholesterol complex in liposomal membranes was monitored by using a fluorescence probe, Ch-PnA. The results are presented in Fig. 4 as Stern-Volmer plots (the dependence of the ratio of the initial fluorescence intensity [F_0] to a fluorescence intensity at any given time point after the addition of AmB [F] on the AmB concentration). We found that low concentrations of AmB (0.06 to 6 μM) quenched the fluorescence of Ch-PnA (increased the F_0/F ratio). As can be seen, the plots deviate from linearity and display a saturation pattern. The deviation from linearity in Stern-Volmer plots suggests that the quenching is not the result of thermal collisions between molecules, but is the result of the complex formation between Ch-PnA and AmB. The cholesterol moiety of Ch-PnA was essential for the formation of the complex since the same concentrations of AmB did not quench the fluorescence of free PnA (data not shown).

The data on the quenching of Ch-PnA fluorescence by AmB were used to select the concentration of cholesterol in liposomes that turn most of the added AmB to the cholesterol-bound state. To this end, we studied the effects of different cholesterol concentrations in the liposomal membrane on the ability of AmB to quench the Ch-PnA fluorescence (Fig. 4).

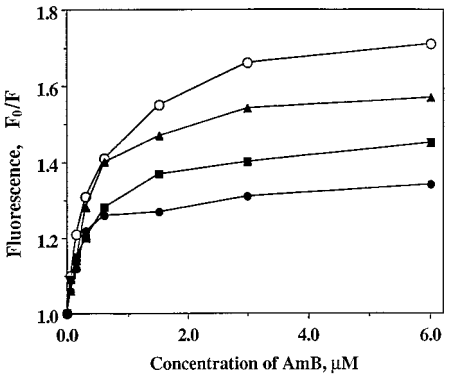

FIG. 4. Stern-Volmer plots of Ch-PnA fluorescence quenching by AmB in cholesterol-free and cholesterol-loaded liposomes. The incubation mixture contained 0.1 M phosphate buffer (pH 7.4 at 37°C), 100 mM deferoxamine mesylate, and 3 mM Ch-PnA incorporated into liposomes (0.50 mM total lipids). The cholesterol concentration in liposomes was 0 mol% (open circles), 5 mol% (solid triangles), 10 mol% (solid squares), and 20 mol% (solid circles). The AmB concentration was 0, 0.06, 0.15, 0.3, 0.6, 1.5, 3.0, and 6.0 μM. F_0 and F, fluorescence intensities without AmB and in the presence of AmB (as indicated), respectively.

We found that cholesterol at 20 mol% (0.1 mM) prevented, to a large extent, the ability of AmB to quench the fluorescence of Ch-PnA.

Effects of AmB on AMVN-induced oxidation of PnA. In these experiments, AMVN-induced changes in the PnA concentration were assayed by HPLC and directly by measuring PnA fluorescence. It is noteworthy that the time course of PnA oxidation in the absence of AmB was the same by both methods (Fig. 5). Incubation of liposomes with 1 mM AMVN for 60 min caused oxidation of 80% PnA in liposomes. Both concentrations (3 or 10 μM) of AmB incorporated into liposomes significantly reduced the rate of PnA oxidation by AMVN, as evidenced by both fluorescence ($P < 0.001$) and HPLC mea-

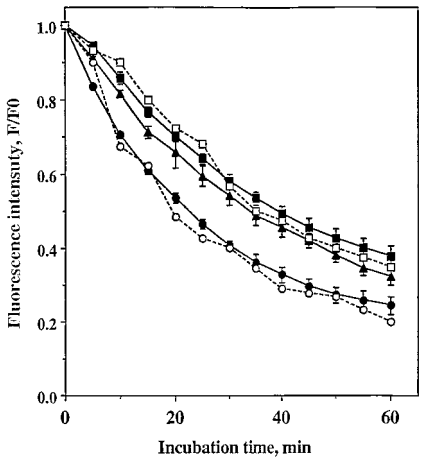

FIG. 5. Time course of AMVN-induced oxidation of PnA in liposomes as detected by spectrofluorometry and HPLC. The reaction mixture contained 0.1 M phosphate buffer (pH 7.4 at 37°C), 100 mM deferoxamine mesylate, 3 mM PnA incorporated into liposomes (0.50 mM total lipids), and 1 mM AMVN. solid circles, solid triangles, and solid squares, spectrofluorometric assay; open circles and open squares, HPLC assay. Circles, control; solid triangles, plus 3 mM AmB; squares, plus 10 μM AmB. The results of the spectrofluorometry assay are given as means ± SEMs ($n = 4$). The statistical analysis was performed as described in the legend to Fig. 2 and yielded a significant difference for every time point between both AmB-free liposomes and AmB-loaded liposomes, with $P < 0.05$.

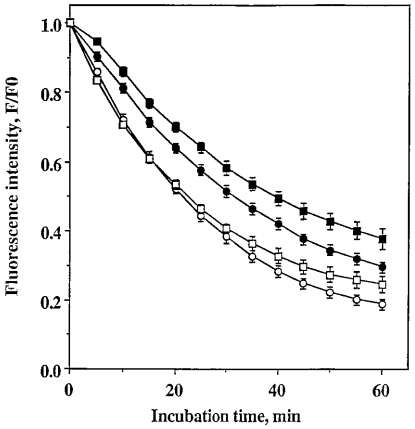

FIG. 6. Effect of AmB on the time course of AMVN-induced oxidation of PnA in cholesterol-free and cholesterol-loaded liposomes as detected by the spectrofluorometric assay. Squares, cholesterol-free liposomes; circles, cholesterol-loaded liposomes (20 mol% cholesterol among the total lipids). The reaction mixture contained 0.1 M phosphate buffer (pH 7.4 at 37°C), 100 mM deferoxamine mesylate, 3 mM PnA incorporated into liposomes (0.50 mM total lipids), and 1 mM AMVN. Open symbols, control; solid symbols, plus 10 mM AmB. The results are means ± SEMs ($n = 4$) ($P < 0.05$). The statistical analysis was performed as described in the legend to Fig. 2 and yielded significant differences for every time point between cholesterol-free or cholesterol-loaded liposomes and AmB-free liposomes as well as AmB-loaded liposomes, respectively, with $P < 0.05$. In addition, the differences for every time point between AmB-loaded liposomes under conditions with and without cholesterol were also significant, with $P < 0.05$.

surements. Statistical analysis to estimate the effects of AmB confirmed an inhibiting effect of AmB ($P < 0.05$) for every time point through the PnA oxidation compared to the effect of PnA alone (Fig. 5). Furthermore, the effect was concentration dependent: 10 μM AmB inhibited PnA oxidation more effectively than 3 μM AmB.

Effects of AmB on oxidation of PnA in the presence of cholesterol. In a second series of experiments with liposomes, we examined the effect of AmB on the oxidation of PnA in the presence of cholesterol. In the absence of AmB, cholesterol (20 mol% or 0.1 mM) did not significantly affect PnA oxidation during the initial 30 min of the reaction. At longer incubation times, cholesterol slightly (but significantly [$P < 0.05$]) increased the rate of PnA oxidation by AMVN in liposomes (Fig. 6). During the initial 30 min of the reaction, cholesterol significantly diminished (but did not eliminate [$P < 0.05$]) the inhibitory effect of AmB on AMVN-induced oxidation of PnA in liposomes (Fig. 6). These results indicate that AmB, either in free form or in complex with cholesterol, did not exert any prooxidant activity.

DISCUSSION

In the present study, we attempted to evaluate the antioxidant and prooxidant effects of AmB using a well characterized in vitro system in which we used a natural fatty acid with four conjugated carbon-carbon double bonds, PnA, as a reporting molecule extremely sensitive to oxidation (7). The two major findings of the study are that (i) AmB did not enhance oxidation of PnA but rather, vice versa, it caused an inhibitory effect in both hydrophobic membrane domains and in water-soluble complexes of PnA with hSA and (ii) this inhibitory effect was a characteristic of free AmB because cholesterol diminished the inhibitory effect of AmB on PnA oxidation.

Several studies have previously implicated autoxidation of AmB, and consequently its ability to cause oxidative stress as the mechanism(s) of cytotoxicity associated with the therapeutic effectiveness of AmB (2, 3, 18). Indirect support for this hypothesis is provided by the evidence of protection against AmB-induced toxicity afforded by antioxidant enzymes or antioxidant compounds (2). Brajtburg et al. (2) suggested that oxidative damage might be involved in AmB-induced hemolysis of erythrocytes. However, Cutaia et al. (5) found no evidence of oxidative stress associated with the AmB-induced injury of endothelial cells. Our results extend this observation to suggest that rather than a prooxidant, AmB is in fact an antioxidant.

Radical scavenging activity of AmB. AmB demonstrated an ability to effectively scavenge peroxyl radicals generated by both lipid-soluble (AMVN) and water-soluble (AAPH) initiators of peroxyl radicals. This suggests that AmB acts as an antioxidant by preventing oxidation of PnA. Since the conjugated double-bond system in PnA is more susceptible to oxidation than the methylene-interrupted double bonds in polyunsaturated membrane phospholipids (21), it seems highly unlikely that AmB is able to propagate lipid peroxidation in biomembranes. The antioxidant effects of AmB were different from those of vitamin E (α-tocopherol) (13) or other lipid-soluble phenolic chain-breaking antioxidants (e.g., ubiquinol and probucol) (20) in that it did not cause a lag period in the peroxidation of PnA. Rather, AmB inhibited PnA oxidation by competing for peroxyl radicals in a way similar to the effect of β-carotene (13, 19). Tsuchiya et al. (20) have reported that the conjugated double-bond structure is critical for the scavenging activities of carotenoids and retinoids. β-Carotene, AmB, and retinoids have 11, 7, and 5 conjugated double bonds (9), respectively. Not surprisingly, AmB's antioxidant effectiveness is superior to those of retinoids but significantly lower than those of carotenoids. β-Carotene is known to be reactive toward peroxyl radicals, competes with the allylic hydrogens of polyunsaturated fatty acids in membranes, and produces a carotenyl radical (13, 19). In the absence of oxygen, the latter is an efficient chain terminator. In the presence of oxygen, the carotenyl radical reacts with oxygen to yield a chain-propagating species, the β-carotene peroxyl radical, which triggers further oxidations (12, 14). Thus, β-carotene acts as an effective chain-breaking antioxidant specifically at low partial pressures of oxygen (19). AmB is also likely to act as an effective chain-breaking antioxidant.

Lamy-Freund et al. (8) showed that at high concentrations AmB autoxidation gave rise to free radicals detectable by spin traps. In contrast, Rickards et al. (16) failed to detect free radicals using low concentrations of polyene antibiotics. Our results indicated that at a higher concentration (10 μM) AmB protected PnA against AMVN-induced oxidation more effectively than at a lower concentration (3 μM). This might not be the case if AmB-derived free radicals were effective in propagating PnA peroxidation. Thus, AmB likely acts as an antioxidant rather than a prooxidant in cells experiencing oxidative insult. This does not preclude, however, the possibility that the AmB peroxidation products formed as a result of its radical scavenging activity may exert cytotoxic effects exceeding that of AmB, similar to toxic fragments of β-carotene peroxidation (6, 11, 19).

Cholesterol diminishes the radical scavenging activity of AmB. It is well known that AmB's effects on membranes are mediated by its interactions with sterols (1). We found that cholesterol diminished the inhibitory effect of AmB on AMVN-induced PnA peroxidation during the initial 30 min of the reaction. Whether this effect is mainly due to restrictions on the mobility of the cholesterol-AmB complex (compared to that of free AmB) and its decreased availability to AMVN-

derived peroxyl radicals remains to be elucidated. Since cholesterol can itself intercept free radicals (15), it might compete with both AmB and PnA for AMVN-derived peroxyl radicals. This, however, is not likely to be the case, since in the absence of AmB, cholesterol did not significantly affect PnA oxidation in liposomes during the initial 30 min of incubation. Thus, free AmB acted as an effective scavenger of peroxyl radicals in membranes during the initial oxidation period. Cholesterol-bound AmB also exerted antioxidant activity which was, however, less pronounced than that of free AmB. At longer incubations (>30 min) the diminishing effect of cholesterol was no longer observable. While the exact mechanism(s) responsible for the diminishing effect of cholesterol is still to be elucidated, one may speculate that oxidation of AmB by AMVN-derived peroxyl radicals resulted in the loss of its ability to form complexes with cholesterol but did not eliminate the ability of oxidatively modified AmB to act as a radical scavenger.

Finally, we demonstrated that AmB forms complexes with Ch-PnA in which the PnA fluorescence was quenched. Addition of cholesterol reconstitutes the Ch-PnA fluorescence evidently by competing with Ch-PnA, forming cholesterol-AmB complexes and releasing fluorescent Ch-PnA. This finding offers a unique opportunity to develop fluorescence-based techniques for measurements of free versus cholesterol-bound AmB in membranes and biological fluids. This may be important for evaluating the radical scavenging effects of AmB as well as its other activities dependent on either the free or cholesterol-bound state.

In conclusion, our results directly demonstrate that free AmB or cholesterol-bound AmB may act as an antioxidant. This definitely contradicts current opinion on the prooxidant activity of AmB as the basis of its cytotoxicity in vitro.

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