ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1992, p. 262–266
0066-4804/92/020262-05$02.00/0

Vol. 36, No. 2

Influence of Phospholipid/Amphotericin B Ratio and Phospholipid Type on In Vitro Renal Cell Toxicities and Fungicidal Activities of Lipid-Associated Amphotericin B Formulations

VÉRONIQUE JOLY,[1] JACQUES BOLARD,[2] LINE SAINT-JULIEN,[1] CLAUDE CARBON,[1] AND PATRICK YENI[1]†*

Laboratoire d'Etude des Traitements Antiinfectieux, Faculté Xavier Bichat, Université Paris 7 et Institut National de la Santé et de la Recherche Médicale Unité 13, 46 rue Henri Huchard, 75018 Paris,[1] and Laboratoire de Physique et Chimie Biomoléculaire, Centre National de la Recherche Scientifique Unité 198, Tour 22, Université Pierre et Marie Curie, 75252 Paris,[2] France

Received 24 June 1991/Accepted 25 October 1991

We studied the influence of the lipid/amphotericin B (AMB) ratio and the phospholipid type on the in vitro renal cell toxicity and antifungal efficacy of lipid-associated AMB (L-AMB). L-AMB was prepared at one of two different lipid/AMB ratios (1 and 40) by incubating AMB with empty small unilamellar vesicles, made from one of three different phospholipids: dipalmitoyl-, dimirystoyl-, and distearoylphosphatidylcholine (DPPC, DMPC, and DSPC, respectively). Renal cell toxicity, investigated through an assessment of the Na-dependent uptake of phosphate by proximal tubular cells, and fungicidal effect against *Candida albicans* were studied after 1 h of treatment at 37°C. The amount of unbound AMB present in each L-AMB formulation was studied by use of circular dichroism. At a lipid/AMB ratio of 40, the three lipidic formulations were not toxic for renal cells but were less effective against *C. albicans* than AMB; however, DSPC-AMB, which contained 50% unbound AMB, was more effective against *C. albicans* than DPCC-AMB or DMPC-AMB, containing 0 and 13% unbound AMB, respectively. At a lipid/AMB ratio of 1, the antifungal effects of L-AMB and AMB were similar, whatever the phospholipid used, but only DMPC-AMB remained highly protective against AMB renal cell toxicity, despite the presence of the same amount of unbound AMB (50%) in DMPC-AMB and DPPC-AMB. We conclude that the in vitro activities and renal cell toxicities of different L-AMB formulations are influenced by the phospholipid type and the lipid/AMB ratio. The optimal ratio depends on the phospholipid itself. At a lipid/AMB ratio of 40, the antifungal activity depends mainly on the amount of unbound AMB in the formulation. At a lipid/AMB ratio of 1, the renal cell toxicity also depends on the fluidity of the phospholipid.

Amphotericin B (AMB) is the drug of choice for most systemic fungal infections (6, 18). The cytotoxic mechanism of this compound is thought to rely on its ability to form membrane ion channels, particularly in the presence of sterols. The greater affinity of AMB for ergosterol-containing membranes (fungal cells) than for cholesterol-containing membranes (mammalian cells) forms the basis of the selective toxicity of the drug (1, 3, 5, 25). However, the clinical use of AMB remains highly limited by its toxicity (18, 21).

The incorporation of AMB into liposomes has been shown to reduce in vivo (10, 17, 19, 22) and in vitro (11–13, 15) toxicities. The increased in vivo tolerance of liposomal AMB infusions as compared with deoxycholate-associated AMB enables the administration of larger doses of the drug, thereby enhancing its therapeutic effect, as shown by many experimental studies (17, 23, 24) and the first clinical results (16, 20). However, it is difficult to compare the data on liposomes reported by different laboratories because the methods of production, types of lipid, vesicle size, and concentrations of sterols, etc., differ from one laboratory to another. Since the extent of protection against AMB toxicity afforded by liposomes depends on the type of liposomes used, it is not surprising that conflicting data about the effect of liposomes on the in vitro antifungal activity of AMB have been described. In some studies, lipid-associated AMB (L-AMB) was as effective as AMB against fungal cells (7, 8), whereas in others, liposomes reduced the in vitro antifungal activity of AMB (13).

The best formulation of L-AMB, in terms of phospholipid composition and lipid/AMB ratio, remains to be determined. For instance, it was shown that lowering the lipid/AMB ratio led to lipid-stabilized AMB aggregates with dramatically attenuated AMB toxicity for mammalian cells but not fungal cells (9). In vitro screening of toxicity and efficacy should be helpful in determining the most satisfactory formulation, which would then merit further in vivo evaluation. Since the main target of AMB toxicity in vivo is the kidney, a model of toxicity involving renal tubular cells in cultures might be more appropriate than a model involving other mammalian cells, such as erythrocytes or leukocytes, for the in vitro assessment of AMB toxicity. The aim of our study was to compare in vitro the renal tubular cell toxicities and candidacidal activities of L-AMB formulations made with each of the phospholipids dipalmitoylphosphatidylcholine (DPPC), dimirystoylphosphatidylcholine (DMPC), and distearoylphosphatidylcholine (DSPC) at two different lipid/AMB ratios, 1 and 40.

(This work was presented in part at the 30th Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, Ga., 21 to 24 October 1990 [V. Joly, J. Bolard, L. St. Julien, C. Carbon, and P. Yeni. 1990. Program. Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 758].)

* Corresponding author.
† Present address: Service de Médecine Interne, 10 ème étage, Hôpital Bichat, 46, rue Henri Huchard, 75018 Paris, France.

MATERIALS AND METHODS

Materials. AMB in powdered form was obtained as a gift from Squibb (Neuilly-sur-Seine, France). Ethanolamine, insulin, transferrin, hydrocortisone, triiodothyronine, sodium selenite, Percoll, collagenase type I, collagen type 1, DMPC, DPPC, and DSPC were purchased from Sigma Chemical Co. (St. Louis, Mo.). Dimethyl sulfoxide (Me_2SO) was obtained from Merck (Darmstadt, Germany). Radiolabeled tracer $K_2H^{32}PO_4$ was obtained from New England Nuclear (Boston, Mass.). Culture media were obtained from Flow Laboratories (Irvine, United Kingdom). Liquid Sabouraud medium was obtained from Diagnostics Pasteur (Marnes-la-Coquette, France).

Preparation of AMB and L-AMB solutions. (i) AMB. AMB (powder) was stored at 4°C and prepared before each experiment as follows. AMB was first dissolved at 30 mM in Me_2SO and then diluted in buffer containing 10 mM sodium phosphate and 150 mM NaCl (PBS) (pH 7.4).

(ii) L-AMB. DMPC, DPPC, and DSPC were used without further purification, and small unilamellar vesicles were prepared by sonication as previously described (13). The phospholipid was dissolved in chloroform, and the solvent was evaporated under nitrogen. PBS was added to the flask, and the sample was sonicated above the transition temperature of the phospholipid used until the suspension was clarified. AMB was dissolved first at 30 mM in Me_2SO and then in PBS. The amount of AMB in PBS added to suspensions of vesicles was calculated to yield a molar phospholipid/AMB ratio of 40 or 1. Since the structure of the vesicles after the incorporation of AMB was not studied microscopically, the conformations of the preparations could not be ascertained, especially in the case of a low phospholipid/AMB ratio. Therefore, the name L-AMB was preferred over the name liposomal AMB in the present study.

Binding of AMB to small unilamellar vesicles. For the phospholipid/AMB ratio of 1 or 40, binding was measured in PBS at 37°C after 10 min of incubation of 5 μM AMB with 5 or 200 μM phospholipid, respectively. We used circular dichroism as previously described (14). In fact, although the spectrum of absorbance was between 300 and 450 nm, the free- and bound-drug spectra overlap, so that there is no wavelength at which only one of the species absorbs. In contrast, AMB circular dichroism presents features which allow monitoring of the amount of drug remaining free in the presence of liposomes. At the concentration of AMB studied (5 μM), the circular dichroism spectrum of free AMB consisted of an intense dichroic doublet centered at 340 nm. Because the spectrum of AMB bound to pure phospholipid vesicles did not consist of the dichroic doublet centered at 340 nm, the intensity of this doublet permitted us to determine the amount of unbound AMB present in a formulation.

Toxicity study. (i) Cell cultures. Kidneys were excised under anesthesia from male New Zealand White rabbits (Evic Ceba, Blanquefort, France) (800 to 1,000 g), and primary confluent monolayers were grown from proximal tubule fragments in serum-free medium as previously reported (4, 11). The cultures became confluent in 5 to 6 days. All experiments reported were carried out on day 7.

(ii) Exposure of cells to free AMB and L-AMB. The culture medium was changed on the day before experiments. On day 7, cells grown to confluence were incubated with fresh culture medium containing the appropriate concentration of free AMB or L-AMB for 1 h. The final concentration of Me_2SO in the supernatant of treated wells was less than 0.14%.

(iii) P_i uptake. P_i uptake was chosen as a marker of early tubular cell toxicity as described previously (11). AMB was removed after the 1-h incubation by washing, and P_i uptake was measured as previously described (4, 11). In brief, P_i uptake was determined at 37°C in a buffered uptake solution of the following composition (millimoles per liter): NaCl, 137; KCl, 5.4; $CaCl_2$, 1; $MgSO_4$, 1.2; and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), 15. Choline chloride replaced sodium chloride in the sodium-free solution. Cells were washed twice with 0.5 ml of the appropriate ice-cold uptake solution per well and then incubated for 10 min in the presence of $K_2H^{32}PO_4$ (0.5 μCi/ml) and 0.1 mM unlabeled KH_2PO_4. We verified previously that P_i uptake increased linearly with time up to 10 min. Uptake was stopped by washing the cells twice with 1 ml of ice-cold buffer (137 mM NaCl, 15 mM HEPES [pH 7.4]) per well. The cells were solubilized in 0.5% Triton X-100 (250 μl per well), and the radioactivity in aliquots was counted by liquid scintillation. Radioactive counts in each sample were normalized with respect to the protein content of each well, determined by the Bradford method (2). Each experimental point was tested in triplicate.

Antifungal activity. A *C. albicans* isolate grown from a patient's blood culture was used. Fungal cells were grown for 18 h in Sabouraud dextrose broth at 37°C, washed three times with PBS, resuspended at 10^7 CFU/ml, and incubated for 1 h at 37°C with the appropriate concentration of free AMB or L-AMB. At the end of the incubation, the cells were washed three times in PBS and appropriate dilutions were made before plating on Sabouraud dextrose agar. Duplicate plate counts were determined after 24 h of culturing at 37°C. Results are expressed as percentages of the survival of a control inoculum. Each experimental point was tested in duplicate.

Statistical analysis. For each treatment, at least three different experiments were performed to test activity and toxicity. For each AMB dose, the different formulations (AMB, DMPC-AMB, DPPC-AMB, and DSPC-AMB) at a given ratio were compared by a one-way analysis of variance and, when allowed by the *F* value, means were computed by Fisher's protected least-significant-difference test with Stat View on a Macintosh S.E. Differences were considered to be significant at $P < 0.05$.

RESULTS

Measurement of the amount of AMB bound to lipids. At a total AMB concentration of 5 μM and a 40:1 lipid/AMB ratio, the amounts of free AMB in the L-AMB formulations were 2.5, 0, and 0.6 μM for DSPC-AMB, DPPC-AMB, and DMPC-AMB, respectively. At a total AMB concentration of 5 μM and a 1:1 lipid/AMB ratio, the amounts of free AMB in the L-AMB formulations were 4.7, 2.5, and 2.3 μM for DSPC-AMB, DPPC-AMB, and DMPC-AMB, respectively.

Renal tubular cell toxicity. At a lipid/AMB ratio of 40, the three lipidic formulations exhibited the same complete protective effect against AMB toxicity, as shown by the absence of P_i uptake inhibition. Mean P_i uptake by cells treated with any L-AMB was not significantly different from the mean control value measured in untreated cells but differed significantly at the three concentrations tested from the mean value measured in cells treated with free AMB ($P < 0.001$) (Fig. 1A). At a lipid/AMB ratio of 1, DSPC-AMB was as toxic as free AMB. DPPC-AMB and DMPC-AMB both reduced the P_i uptake inhibition due to AMB but, for a given AMB concentration, DMPC-AMB was significantly less

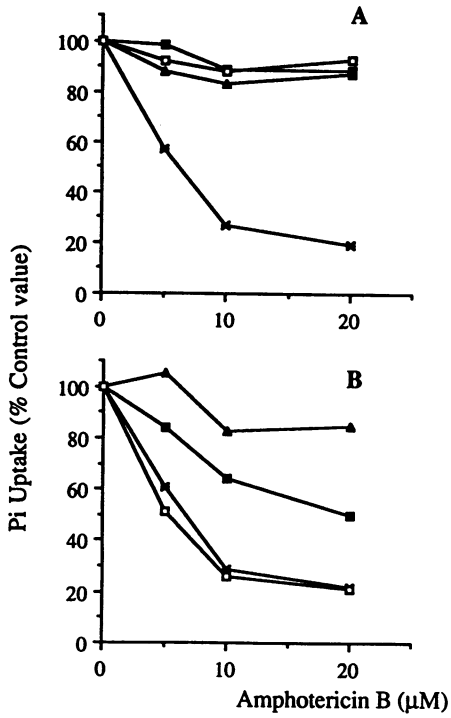

FIG. 1. Effect of AMB and L-AMB on Na^+-dependent uptake of P_i by renal tubular cells. The results are expressed as percentages of control values, obtained through measurement of P_i uptake in untreated cells. Data are represented as the means of three different experiments in which duplicates were tested. The phospholipid/AMB ratios were 40 (A) and 1 (B). Symbols: ×, free AMB; ▲, DMPC-AMB; ■, DPPC-AMB; □, DSPC-AMB.

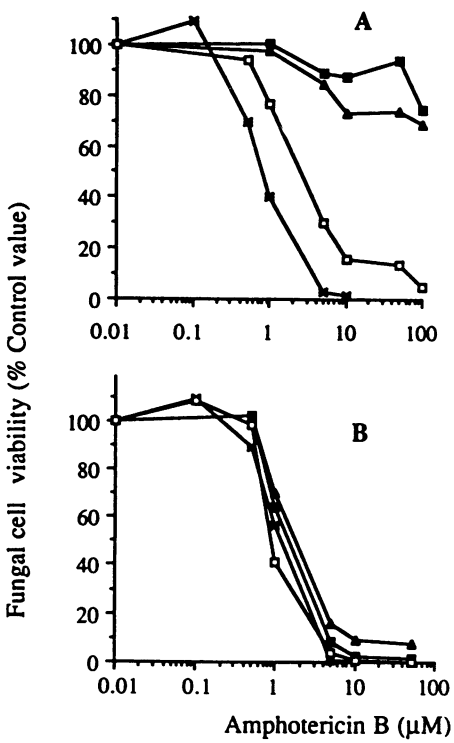

FIG. 2. Effect of AMB and L-AMB on *C. albicans* viability. The results are expressed as percentages of control values. Data are represented as the means of three different experiments in which duplicates were tested. Ratios and symbols are as defined in the legend to Fig. 1.

toxic than DPPC-AMB ($P < 0.05$ for 5 μM L-AMB and $P < 0.01$ for 10 and 20 μM L-AMB) (Fig. 1B). Empty liposomes were atoxic for renal tubular cells, whatever the phospholipid used (data not shown).

Antifungal effect. At a lipid/AMB ratio of 40, each L-AMB formulation was significantly less efficient than the same concentration of free AMB ($P < 0.05$ for DSPC-AMB and $P < 0.01$ for DMPC-AMB and DPPC-AMB) at all the concentrations tested from 1 μM up (Fig. 2A). However, DSPC-AMB was significantly more toxic for fungal cells than DPPC-AMB or DMPC-AMB ($P < 0.01$) at between 5 and 100 μM AMB; 70 and 95% decreases in fungal cell viability were observed after exposure to 5 and 100 μM DSPC-AMB, respectively. In contrast, neither of the other lipidic formulations induced more than 30% lethality, even at the highest AMB concentration tested. On the other hand, decreasing the ratio to 1 restored the antifungal effect of all the L-AMB formulations tested (Fig. 2B). Although not significant (F test), slight differences were noted; the concentrations needed to induce a 50% decrease in fungal cell viability were 0.8-fold (DSPC-AMB), 2-fold (DPPC-AMB), and 2.5-fold (DMPC-AMB) those of free AMB. Empty liposomes were atoxic for fungal cells, whatever the phospholipid used (data not shown).

DISCUSSION

Liposomal AMB or, in a broader sense, L-AMB, has been proven to be safe and effective in the treatment of systemic fungal infections (16, 20). However, the formulations used differ in terms of composition, vesicle size, and lipid/AMB ratio. These differences explain in part the variability of the results which have been reported concerning the reduced toxicity and intrinsic antifungal activity of liposomal AMB, especially in vitro (8, 12, 13). Comparison of different formulations in the same system might lead to a better understanding of the mechanisms involved in the cellular toxicity of L-AMB and would be useful in defining which parameters should be controlled in the preparation of an optimal formulation.

Among the possible crucial parameters, the lipid/AMB ratio appears to vary widely from one study to another or even within the same study. The influence of this ratio on the structure and thus on the activity of L-AMB has recently been studied by Janoff et al. (9). In their study, it appeared that, despite major alterations in the L-AMB complex structures, the beneficial effect of lipids was retained and even enhanced when the ratio was decreased to 1.

In the present study, we evaluated in vitro the renal toxicities and fungicidal effects of six different L-AMB formulations. Our model of tubular cells in cultures was used previously to examine the mechanisms involved in AMB renal tubular cell toxicity (11). We showed that the alteration of membrane-related functions, i.e., sodium-dependent uptake, was an early event in AMB nephrotoxicity. Thus, P_i uptake was selected here as the marker of the harmful effects of AMB on mammalian cells. The in vitro antifungal activity was evaluated as the candidacidal effect, not the fungistatic effect, of AMB. In fact, AMB is the only strongly fungicidal antifungal agent clinically available (18). This property distinguishes it from another antifungal drugs and correlates

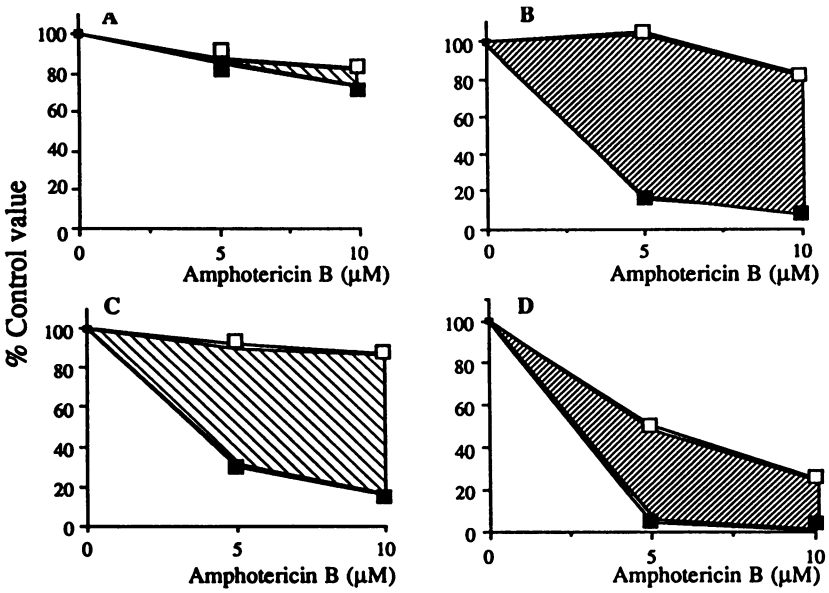

FIG. 3. Comparison of the renal toxicities (□) and antifungal activities (■) of DMPC-AMB (A and B) and DSPC-AMB (C and D) formulations. The therapeutic index is represented by the area bounded by the two curves. The phospholipid/AMB ratios were 40 (A and C) and 1 (B and D).

with in vivo observations (17a). Thus, this severe criterion of efficacy, reinforced by the short time of exposure (1 h), was chosen to discern the existence of possible differences between the activities of the different treatments. Experiments were performed at 37°C, the in vivo temperature, to take into account the influence of temperature on the physical state (gel or fluid state) of the phospholipids. Since AMB incorporated into unsaturated phospholipid liposomes is much more potent in inducing ion fluxes in erythrocytes than AMB incorporated into saturated phospholipid liposomes (12), we used three saturated phospholipids with the same head group (choline). The only differences were the length of the saturated carbon chain (14 carbon atoms in DMPC, 16 in DPPC, and 18 in DSPC) and the transition temperature (22°C for DMPC, 41°C for DPPC, and 58°C for DSPC). With these three phospholipids, it was shown that the AMB binding affinity for vesicles made from one of these lipids decreased in the order DPPC > DMPC > DSPC (14).

The cellular toxicities of the L-AMB formulations could easily be altered by small differences in phospholipid structures and by variations in the lipid/AMB ratio. At a ratio of 40, all the L-AMB formulations were less active than free AMB. This observation was not in agreement with the findings reported in other studies (7, 8, 22). This lack of agreement could be due either to the severe criterion of activity that we chose, unlike some experiments in which an inhibitory (7, 22) or a killing (8, 22) effect after 24 h of exposure was used, or to the lower value, i.e. 10, of the lipid/AMB ratio used previously (22). However, it agrees with our previous results obtained with another strain of *C. albicans* at a lipid/AMB ratio of ≥20 (13). Although the L-AMB formulations showed a lower level of activity than free AMB, they differed from one another. These differences in activity reflected the differences in free AMB content among the three L-AMB formulations measured by circular dichroism. At a total AMB concentration of 5 μM, the concentrations of free AMB were 0, 0.06, and 2.5 μM in the presence of DPPC, DMPC, and DSPC, respectively. In fact, DSPC-AMB was the most active against fungal cells, as previously reported (13). Despite these different levels of activity, the three formulations were not toxic for renal tubular cells, perhaps because of either a lower affinity of free AMB for cholesterol-containing membranes than for ergosterol-containing membranes or an intrinsic protective effect of the phospholipids present in the medium against AMB toxicity, due to an alteration of the renal cell plasma membrane.

At a lipid/AMB ratio of 1, all L-AMB formulations were fungicidal. At this ratio, the three different L-AMB formulations provided clear, but unequal, levels of protection. Here again, the amount of free AMB present in the lipid formulations, as determined in the present study, correlated well with the observed activity. An increase in the free AMB content might be responsible for a higher renal tubular cell toxicity. Interestingly, although DMPC did not show a higher avidity for AMB than DPPC at a low lipid/AMB ratio, it was more protective against AMB-induced renal P_i uptake inhibition. Since, contrarily to those of the other phospholipids used, the transition temperature of DMPC is below 37°C, fluidity could be an important factor involved in the protection afforded by lipids at low concentrations. This property previously was not considered to be important for lipid/AMB ratios of ≥10 (11, 13).

Our results show that fungicidal in vitro activities and renal cell toxicities of different L-AMB formulations are influenced by the phospholipid type and the lipid/AMB ratio. The optimal value for this ratio depends on the phospholipid itself. In our model, the most interesting lipid/AMB ratios, in terms of increasing the therapeutic index of AMB while minimizing renal cell toxicity, were 40 for DSPC-AMB and 1 for DMPC-AMB (Fig. 3). Such in vitro screening might be helpful in selecting L-AMB formulations before undertaking more extensive in vivo studies. However, direct extrapolation to the in vivo situation is not straightforward, since many other factors interfere with the activity and toxicity of

L-AMB, such as AMB binding to serum proteins or rapid dissociation of L-AMB complexes following administration.

ACKNOWLEDGMENTS

We are indebted to Janet Jacobson for helpful comments.

This work was supported by a grant from the Agence Nationale de la Recherche sur le SIDA.

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