Comparative internalization and recycling of different amphotericin B formulations by a macrophage-like cell line

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The amount of amphotericin B (AmB) associated with cultured murine macrophage-like J774 cells, after incubation with various AmB lipid formulations, was determined by absorption spectroscopy. Large, negatively charged, AmB-containing, multilamellar vesicles and small cholesteryl sulphate-AmB complexes both enhanced the amount of AmB associated with J774 cells at 37°C (up to 500-fold the extracellular concentration). In contrast, AmB-containing, small, negatively charged vesicles (AmBisome), positively charged, oligolamellar vesicles and mixed micelles showed a lower association of the antibiotic with cells, compared with AmB added from a solution in dimethylsulphoxide or Fungizone*. Experiments performed at 4°C showed a large reduction of AmB uptake for AmB preparations and AmB added from a solution in dimethylsulphoxide or Fungizone, suggesting a high percentage of internalization of the antibiotic. Experiments in the presence of cytochalasin B resulted in a decrease of AmB uptake mainly for the preparations of large diameter, suggesting that these formulations were taken up by phagocytosis. A comparative study with Chinese hamster ovary cells, a model of non-phagocytic cells, showed a reduction in the take up of AmB. This reduction was always more marked when AmB was incorporated in lipid formulations. On the other hand, accumulation of the antibiotic in J774 cells was shown to be followed by its release from the cells in an unbound form, the extent of release depending on the type of vector used. The results suggest that in some cases macrophages can be considered as reservoirs of antibiotic, releasing free AmB in the medium.

Introduction

Amphotericin B (AmB), is considered to be the antifungal agent of choice for the treatment of disseminated fungal infections in immunocompromised patients despite its toxicity. In fact, the relatively low selectivity of AmB for fungal cells remains a dose-limiting factor. Recent advances have focused on the improvement of its therapeutic index, through reduction of AmB toxicity by its incorporation in lipid carriers. Its high affinity for lipids renders AmB suitable for intercalation into colloidal preparations (liposomes, micelles and complexes). Some of these have been tested in vitro, on fungal and mammalian cells, or in vivo, mainly in mice (Brajtburg et al.,

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519
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Preliminary human clinical trials and experiments in animal models showed an improved efficacy of various AmB lipid preparations, as compared with conventional iv AmB (Janknegt et al., 1992). This improvement was almost always related to the reduction of toxicity and hence to the higher doses of AmB which could be administered.

Although the mechanism of action of liposomal AmB is not fully understood, one hypothesis suggests that liposomes, which are naturally removed from the circulation by phagocytic cells of the mononuclear phagocyte system (MPS) (Gregoriadis, 1988), thus accumulate in commonly infected organs (liver, spleen, lungs) (Lopez-Berestein, Rosenblum & Mehta, 1984). Since macrophages play a central role in host defence against invading fungi or parasites, and AmB is known to be active against several intraphagocytic pathogens such as *Candida albicans* (Tollemar, Ringdén & Tyden, 1990) or *Leishmania* spp. (Alving, 1983), we decided to compare the interactions between AmB lipid carriers and these phagocytes. Depending on the size of the vesicles, internalization by phagocytic cells occurs by an endocytic process, in some cases receptor-mediated, for soluble and insoluble material, and by phagocytosis for larger insoluble particles (> 1 μm) (Besterman & Low, 1983). We tested eight formulations of AmB described in the literature, five of which have been investigated in human clinical trials (Sculier et al., 1988; Lopez-Berestein et al., 1989; Kan et al., 1991; Ringdén et al., 1991; Sanders et al., 1991).

**Materials and Methods**

*Amphotericin B formulations (Table I)*

*Amphotericin B.* AmB powder was a generous gift from Squibb France (Neuilly, France). Stock solutions in dimethylsulphoxide (DMSO) (Prolabo; Paris, France) were freshly prepared at a concentration of 1 mg/mL, so that after dilution in the culture medium, even the largest amount of AmB added corresponded to only 2% DMSO v/v.

*L-AmpB5, L-AmpB10, L-AmpB33.* AmB lipid mixtures of dimyristoyl phosphatidylcholine/dimyristoyl phosphatidylglycerol/AmB (molar ratios 7:3:0.5 or 1 or 3) were prepared by rehydration of dried lipid films (Lopez-Berestein et al., 1989). All lipids were obtained from Sigma (Saint Louis, USA).

*AmB-liposomes.* These vesicles composed of egg yolk phosphatidylcholine/cholesterol/stearylamine/AmB (molar ratio 4:3:1:0.5) were prepared by solvent dispersion as described by Stainmesse et al. (1990).

*AmBisome.* This commercially available formulation, consisting of small unilamellar liposomes was a gift from Vestar France (Rungis, France). It was composed of hydrogenated soya phosphatidylcholine/cholesterol/distearoyl phosphatidylglycerol (molar ratio 2:1:0.8:0.4), and was resuspended in isotonic saline solution by hand shaking before use.

*Cholesteryl sulphate-AmB complexes.* These colloidal dispersions were prepared by solvent dispersion according to Abra (1991).

*AmB mixed micelles.* These were prepared by co-precipitation of egg yolk phosphatidylcholine and deoxycholate bile salts (EDAM 20, molar ratio 1:14.8:5.4) or glycocholate (EGAM 20, molar ratio 1:14.8:12.5) as described previously (Brajtburg et al., 1992).
The concentrations of AmB in the above preparations were determined spectrophotometrically at 407 nm after 100-fold dilution in methanol (ε_{407nm} = 150000 mol^{-1} cm^{-1}).

**Cell lines and culture conditions**

Fibroblast-like Chinese hamster ovary cells (CHO-K1) were grown in monolayers in humidified air with 5% CO_{2} at 37°C in 25 cm^{2} Petri dishes (Nunc; Roskilde, Denmark) containing 10 mL of Ham's F12 (Nissui Pharmaceutical Co; Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS; Bioproduct, Les Ulis, France), L-glutamine (2 mM), penicillin and streptomycin (50 μ/mL) (Sigma, Saint Louis, USA).

The macrophage-like cells J774 were grown in monolayers in a humidified atmosphere containing 5% CO_{2} at 37°C in 140 cm^{2} Petri dishes (Nunc) containing 10 mL of Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 μ/mL), and streptomycin (50 μ/mL). For experiments, cells were harvested with trypsin (0.05%) and aliquoted 24 h before use (3 × 10^{6} cells per 7 cm^{2} plastic culture dishes, corresponding to 50 μg of protein/mL).

**Interaction of AmB formulations with cells**

**Uptake experiments.** AmB preparations were preincubated for 30 min in DMEM, 10% FBS, before adding to the cells, to allow binding to serum proteins. Cells were incubated in the presence of various AmB concentrations (1–100 μM) for 1, 2, 3, 4 or 5 h at 37°C to evaluate total association of AmB with cells or at 4°C to evaluate the adsorbed fraction, with or without 6 μM cytochalasin B (Sigma). The cells were then rapidly cooled and washed four times with Hepes buffer, calcium- and magnesium-free, pH 7.4 (HCMF) to remove unbound AmB. Cells were lysed with Triton X100 1% for 20 min at 37°C.

**Measurement of AmB release from the cells.** After a 2 h incubation, cells were extensively washed with HCMF. Control cells were treated with TX (At = 0). For the other batches, 1 mL of DMEM, containing 10% FBS, pre-warmed at 37°C was added and the cultures were incubated for a further 1, 2, 3 or 4 h. The cells then were rinsed twice with ice-cool HCMF and treated with TX as described above. In some cases, to determine under which form (free or bound to lipids) AmB was released from the cells, a further incubation was performed in HCMF containing 2% FBS (instead of 10% as in the previous experiments) to allow better detection of AmB, since the medium and serum absorb light in the same region of wavelength as does AmB.

**Measurement of cell-associated AmB.** After the measurements of uptake and release, the total polyene concentrations were measured by measurement of absorption, using a blank of AmB-free cells in TX, with a Cary 212 spectrophotometer (Varian SA, Orsay, France). Antibiotic concentrations were calculated from the optical density measured at 413 nm, with the molar extinction coefficient (ε) of AmB in TX at 413 nm taken as 100000 mol^{-1} cm^{-1}. These conditions were determined after recording the absorption spectra of various concentrations of AmB in the presence of TX and were not significantly modified by the presence of lysed cells. The results are expressed as nmol of AmB per mg of cell protein (Peterson, 1977). In order to calculate intracellular concentrations of AmB, it was necessary to determine the cellular volume per milligramme of protein. Considering the diameter of J774 cells (mean 12 μm), the
<table>
<thead>
<tr>
<th>AmB preparations</th>
<th>Composition</th>
<th>Structure and size (μm)</th>
<th>Bio availability relative to Fungizone&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Clinical trials references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungizone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>deoxycholate/AmB 7:3</td>
<td>micelles &lt;0.4</td>
<td>=</td>
<td>(numerous)</td>
</tr>
<tr>
<td>L-AmpB5 and L-AmpB10</td>
<td>DMPC/DMPG/AmB 7:3:0.5/7:3:1 negative</td>
<td>multilamellar vesicles + sheets 1-6</td>
<td>&lt;</td>
<td>Lopez-Berestein et al. (1989)</td>
</tr>
<tr>
<td>&quot;AmB-Lipid Complex&quot; (ABLC) = L-AmpB33</td>
<td>DMPC/DMPG/AmB 7:3:3 negative</td>
<td>sheets 1.6-11</td>
<td>&lt;</td>
<td>Kan et al. (1991)</td>
</tr>
<tr>
<td>AmB-liposomes</td>
<td>EPC/cholesterol/stearylamine/AmB 4:3:1:0.5 positive</td>
<td>oligolamellar vesicles 0.2-0.3</td>
<td>&gt;</td>
<td>Sculier et al. (1988)</td>
</tr>
<tr>
<td>AmBisome</td>
<td>hydrogenated soyaPC/cholesterol/di-stearoylPG/AmB 2:1:0.8:0.4 negative</td>
<td>small unilamellar vesicles 0.06</td>
<td>&gt;</td>
<td>Ringden et al. (1991)</td>
</tr>
<tr>
<td>EDAM20</td>
<td>EPC/deoxycholate/AmB 14.8:5.4:1 neutral</td>
<td>mixed micelles ND</td>
<td>ND</td>
<td>not available</td>
</tr>
<tr>
<td>EGAM20</td>
<td>EPC/glycocholate/AmB 14.8:12.5:1 neutral</td>
<td>mixed micelles ND</td>
<td>ND</td>
<td>not available</td>
</tr>
<tr>
<td>&quot;AmB-Colloidal Dispersion&quot; (ABCD)</td>
<td>cholesteryl sulphate/AmB 1:1</td>
<td>discs 0.12</td>
<td>&lt;</td>
<td>Sanders et al. (1991)</td>
</tr>
</tbody>
</table>

Names and abbreviations are those used in the cited references.
number of cells per well, and that the Triton lysate contained 50 \( \mu g/mL \) protein, we deduced that 1 mg of J774 cell proteins corresponds to a total cell volume of 5 \( \mu L \). Cellular viability was determined by the trypan blue exclusion assay, using a 1\% trypan blue solution and expressed as a percentage of control.

**Results**

The cellular uptake of free AmB and different AmB formulations was studied as a function of time (up to 5 h), antibiotic concentration (1–100 \( \mu M \)) and temperature (4°C and 37°C), with or without the metabolic inhibitor cytochalasin B (an inhibitor affecting the microfilament system). Uptake at 37°C represented the total cell association, that is binding and endocytosis. At 4°C, endocytosis and phagocytosis were inhibited and, therefore, the measured cell association only represented the adsorption on the membrane.

*Viability of J774 cell line in the presence of free AmB and AmB formulations*

In the absence of serum, J774 cell viability was affected by AmB concentrations > 5 \( \mu M \). AMB 5 \( \mu M \) caused cells to round up; at concentrations higher than 10 \( \mu M \) cells stained with trypan blue; and at AmB 50 \( \mu M \) the cells did not adhere to plastic. J774 cells were more sensitive to AmB-induced toxicity than CHO cells (results not shown).

In the presence of serum, the thresholds of toxicity were shifted to higher concentrations. Figure 1(b) shows that AmB only significantly reduced the viability of J774 cells after a 1 h incubation at 37°C, at concentrations > 10 \( \mu M \) (about 40\% of cells stained by trypan blue after exposure to 50 \( \mu M \)). In contrast, no cellular toxicity was seen with the lipid formulations tested, at concentrations \( \leq 100 \mu M \) AmB.

**Uptake of free AmB and AmB formulations by J774 and CHO cells**

*Effect of AmB concentration.* As shown in Figure 1(a), when AmB in DMSO was incubated for 1 h with J774 or CHO cells, the amount of AmB associated with the cells was concentration dependent. The difference in the amount of AmB associated at 37°C, as compared with that at 4°C, confirms the observation of Vertut-Doi, Ohnishi & Bolard (1994) regarding the internalization of AmB by CHO cells by pinocytosis and endocytosis. It should be noticed that results obtained using AmB alone were comparable with those obtained with deoxycholate-AmB micelles (Fungizone®). This observation is consistent with the fact that dilution of Fungizone® < 5 mM leads to the loss of deoxycholate from the mixture (Lamy-Freund, Ferreira & Schreier, 1989); at concentrations < 5 \( \mu M \) complete dissociation occurs.

With various AmB formulations, after a 1 h incubation with J774 cells, uptake of AmB was also concentration dependent, but the extent of uptake varied considerably between the different formulations (Figure 2(a), (b)). Whereas the uptake of AmB-cholesterol sulphate complex and L-AmpB5 was comparable with that of AmB dissolved in DMSO, much higher levels were obtained with L-AmpB10 and L-AmpB33. In contrast, the uptake of the other studied formulations was lower than that of free AmB.

Table II shows the calculated intra- to extra-cellular concentration ratios for each AmB preparation, at 10, 50 and 100 \( \mu M \) of external AmB. With L-AmpB33, AmB
concentration in J774 cells reached about 15 mM with 50 μM or 100 μM AmB in the external medium, and 7.5 mM with 10 μM AmB, which corresponded to the highest degree of AmB accumulation (ratio 750). The lowest AmB accumulation was observed with AmBisome (ratio always below 10, and AmB concentration <0.2 mM, whatever the extracellular concentration).

Effect of incubation time. Figure 3(a), (b) indicates that almost maximum uptake was reached after a 1 h incubation in most cases, except for L-AmpB 10 and AmB-cholesteryl sulphate complexes.

Inhibiting effects. Table III shows the amount of AmB associated with J774 cells after a 4 h incubation at 37°C, as compared with that after a 4 h incubation at 4°C. As far as the interaction of AmB and different AmB formulations with the murine macrophage-like J774 cells was concerned, adsorption (i.e., values obtained at 4°C) seemed to be a minor process of association, compared with endocytosis, even for AmB alone. The percentage of AmB internalized was in all cases >70% of the total AmB associated with the cells (about 75% for AmBisome, 80% for positively charged AmB-liposomes and L-AmpB33, 85% for AmB-cholesteryl sulphate complexes discs, and >90% for L-AmpB, whatever the final AmB concentration).
Macrophage internalization of amphotericin B

Figure 2. Uptake by J774 cells of the different AmB preparations as a function of the antibiotic concentration, after a 1 h incubation. Cells were incubated at 37°C with: (a) AmB (×) (thick line), L-AmpB5 (▲), L-AmpB10 (■), L-AmpB33 (●) and AmB-cholesteryl sulphate complexes (○); (b) AmB (×) (thick line), EGAM (■), EDAM (●), AmBisome (△), AmB-liposomes (□).

The effect of cytochalasin B, a known inhibitor of phagocytosis, on the uptake of AmB formulations by J774 was studied in order to determine the importance of phagocytosis in the internalization process. Table III compares the uptake of AmB by cytochalasin B-exposed and non-exposed cells, after a 4 h incubation. The uptakes of L-AmpB5 and L-AmpB33 preparations were strongly affected by this inhibitor (62–75% reduction). It should be noted that the uptake of free AmB was reduced by 50% and therefore could not be attributed only to fluid-phase pinocytosis. In the case of CHO cells, no effect of cytochalasin B on internalization was observed (data not shown).

Comparison of AmB internalization between J774 and CHO cells. As shown in Table IV, the amount of AmB internalized (calculated from the total uptake at 37°C minus binding at 4°C) was much lower for CHO cells than for the phagocytic J774 cells.
Table II. Accumulation of AmB in J774 cells. Determination of the uptake was performed after a 4 h incubation at 37°C with various extracellular AmB concentrations

<table>
<thead>
<tr>
<th>AmB preparations</th>
<th>Intra- to extra-cellular concentration ratios ± S.D.</th>
<th>10 μM</th>
<th>50 μM</th>
<th>100 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmB</td>
<td></td>
<td>200 ± 10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L-AmpB5</td>
<td></td>
<td>360 ± 50</td>
<td>135 ± 38</td>
<td>40 ± 11</td>
</tr>
<tr>
<td>L-AmpB33</td>
<td></td>
<td>750 ± 80</td>
<td>315 ± 28</td>
<td>156 ± 25</td>
</tr>
<tr>
<td>AmB-liposomes</td>
<td></td>
<td>17.5 ± 2</td>
<td>6.5 ± 1</td>
<td>7 ± 1.5</td>
</tr>
<tr>
<td>AmBisome</td>
<td></td>
<td>8 ± 2</td>
<td>4 ± 1</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>AmB-cholesteryl sulphate complexes</td>
<td></td>
<td>359 ± 10</td>
<td>136 ± 8</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Mean ± S.D. of triplicate determinations.

Release of AmB from the J774 cell line

The amounts of AmB remaining in or released from cells were determined as a function of time, after a 2 h exposure followed by extensive washing (Figure 4). When macrophages were loaded with L-AmpB5, and L-AmpB33, which allow the highest intracellular accumulation, 20–30% of the total AmB incorporated was released within 2 h, whatever the initial AmB concentration in the loading medium. Under the same conditions, AmB-liposomes and AmB-cholesteryl sulphate complex preparations led to an efflux of 30–40% of the total AmB associated at 10 μM AmB in external medium, and 50–60% for 50 μM AmB. The release from AmBisome loaded cells was almost as quick as that from cells loaded with free AmB, although it represented a very low amount of AmB. In all cases, the amount of AmB left in macrophages, which depended on the AmB concentration in the extracellular medium, was approximately two-fold higher than the amount associated with the plasma membranes. Hence, AmB release cannot only be attributed to AmB exchange with that bound to plasma membrane. It is also interesting to note that qualitative absorption measurements on external medium after the second incubation revealed that AmB was in its free form, with a maximum wavelength at 409 nm. This observation suggests that the AmB released is not bound to the lipid carriers, which were probably disrupted inside the lysosomal compartments of the macrophages.

Discussion

The different liposomal formulations of AmB that are currently under evaluation for use in humans have already shown encouraging efficacies in a variety of invasive fungal infections. However, these liposomes, which have not been tested in randomized comparative trials, might differ in tolerance and efficacy, due to their different structural (e.g. lipid composition and size) and pharmacokinetic (e.g. serum half-life) properties. In fact, clinical data on the AmB formulations obtained with the large and negative DMPC/DMPG (7:3) liposomes indicate a very low plasma concentration and serum half-life, whatever the AmB/lipid molar ratio (5–33 mol%), as compared with conventional AmB. This is thought to be due to a marked, rapid uptake by the reticuloendothelial system (Kan et al., 1991). In contrast, according to other human
Figure 3. Kinetics of uptake by J774 cells of AmB preparations studied at 10 μM AmB final. Cells were incubated at 37°C with: (a) AmB (×) (thick line), L-AmpB5 (▲), L-AmpB10 (■), L-AmpB33 (●), and AmB-cholesteryl sulphate complexes (○); (b) AmB (×) (thick line), EGAM (■), EDAM (●), AmBisome (▲), AmB-liposomes (□).

data, positively charged AmB-liposomes (called "Ampholiposomes") and small negatively charged vesicles (AmBisome) allow a high and sustained AmB blood concentration, which seems to be caused by a slow capture by phagocytic cells (Sculier et al., 1988; Tollemar et al., 1990; Lee et al., 1994). In the present study, we have attempted to determine, by measuring the amount of AmB associated with J774 cells, representative of mononuclear phagocytic cells, whether or not the uptake of these AmB liposomal formulations was modified in comparison with AmB-free liposomes, and whether there was a correlation with their bioavailability in vivo. As the drug is not metabolized in vivo or in vitro (Christiansen et al., 1985), the total AmB associated with cells can be measured directly by absorption.

The important role of fixed macrophages in the cellular pharmacokinetics of free AmB, suggested by the in-vivo results of Edmonds, Davidson & Bertino (1991) is confirmed by our study. AmB was taken up by macrophages to a high extent (about
Table III. Membrane binding versus internalization of AmB and AmB formulations. Percentage of AmB associated with J774 cells at 4°C or in the presence of cytochalasin B, as compared to that associated at 37°C (Incubation time: 4 h)

<table>
<thead>
<tr>
<th>AmB preparations</th>
<th>Concentration (µM)</th>
<th>4°C AmB association (% of control ± s.o.y)</th>
<th>cytochalasin-B (6 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmB</td>
<td>10</td>
<td>22 ± 6</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>L-AmpB5</td>
<td>10</td>
<td>4 ± 1</td>
<td>38 ± 13</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4 ± 1</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>L-AmpB33</td>
<td>10</td>
<td>13 ± 7</td>
<td>35 ± 5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>25 ± 5</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>AmB-liposomes</td>
<td>10</td>
<td>8 ± 3</td>
<td>57 ± 11</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>23 ± 6</td>
<td>70 ± 15</td>
</tr>
<tr>
<td>AmBisome</td>
<td>10</td>
<td>28 ± 1</td>
<td>84 ± 16</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>26 ± 1</td>
<td>89 ± 16</td>
</tr>
<tr>
<td>AmB-Cholesteryl sulphate complexes</td>
<td>10</td>
<td>15 ± 2</td>
<td>71 ± 5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>13 ± 1</td>
<td>63 ± 7</td>
</tr>
</tbody>
</table>

*Means ± s.d. of triplicate determinations

70–80% of total AmB associated with J774 cells was internalized). This result extends to macrophages the observation recently made for CHO cells (Vertut-Doï et al., 1994) and suggests that endocytosis of AmB may be of general significance (Bolard & Vertut-Doï, 1995). Furthermore, this fact gives support to the theory of Ponce & Pechère (1990) and recently Martin et al. (1994), that the antifungal activity of AmB occurs in infected macrophages through a direct interaction between microorganism and drug.

Table IV. Amounts of AmB internalized in J774 and CHO cells after a 4 h incubation. These amounts were obtained from the difference between total AmB uptake at 37°C and AmB binding at 4°C

<table>
<thead>
<tr>
<th>AmB preparations</th>
<th>Concentration (µM)</th>
<th>Amount of AmB internalized in each cell type (nmol/mg of proteins)*</th>
<th>% ratio of AmB internalized in CHO/J774 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmB</td>
<td>10</td>
<td>1.6</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.2</td>
<td>1.2</td>
</tr>
<tr>
<td>L-AmpB5</td>
<td>10</td>
<td>8.7</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>16.1</td>
<td>1.1</td>
</tr>
<tr>
<td>L-AmpB33</td>
<td>10</td>
<td>16.5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>29.6</td>
<td>1.7</td>
</tr>
<tr>
<td>AmB-liposomes</td>
<td>10</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.65</td>
<td>0</td>
</tr>
<tr>
<td>AmBisome</td>
<td>10</td>
<td>0.16</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.37</td>
<td>0.08</td>
</tr>
<tr>
<td>AmB-cholesteryl sulphate complexes</td>
<td>10</td>
<td>7.6</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>15.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Values are the means of two independent experiments each consisting of duplicates; the largest s.d. was < 20% of any value.
Macrophage internalization of amphotericin B

Figure 4. Variation as a function of time of the percentage AmB remaining in J774 cells after extensive washing of cells pre-exposed to various AmB preparations (10 μM (a) or 50 μM (b)) for 2 h. AmB (×) (thick line), L-AmpB5 (▲), L-AmpB33 (●), and AmB-cholesterol sulphate complexes (○), AmBisome (△), and AmB-liposomes (□).

The extent of internalization of the AmB liposomal formulations was fairly consistent with the results of previous studies on the interaction of empty liposomes and macrophages; negatively charged, large liposomes (as found in L-AmpB10 and L-AmpB33) are endocytosed by macrophages to a greater extent (in terms of amount of cell-associated lipids) than neutral and small liposomes (such as AmBisome and AmB-liposomes) (Hsu & Juliano, 1982). Cholesterol is also known to stabilize liposomal bilayers (Allen et al., 1991; Gregoriadis & Davis, 1979) and to have an inhibitory effect on liposomal uptake by the reticuloendothelial system (Patel, Tuzel & Ryman, 1983). We observed a weaker internalization with AmBisome (25 mol% cholesterol) and AmB-liposomes (35 mol% cholesterol) as compared with DMPC/DMPG (7:3) formulations without cholesterol. Internalization of the other colloidal preparations depends on several parameters. The high amounts of AmB taken up in macrophages when the drug is intercalated in disc-like micelles (AmB-cholesterol sulphate complexes)
seem to be due to the high AmB/lipid ratio (50 mol%) as compared with the mixed micelles EGAM 20, EDAM 20 (3 and 4 mol%).

A correlation appeared between our results and those obtained in vivo. The formulations which gave low AmB concentration in macrophages in vitro, like AmBisome and AmB-liposomes, are those which have serum half-lives in vivo greater than Fungizone*; those which promote extensive AmB uptake, like L-AmpB5, L-AmpB10, L-AmpB33 (corresponding to ABLC) and AmB-cholesteryl sulphate complexes (corresponding to ABCD), are found at low concentrations in the circulation.

In contrast, as far as biodistribution data are concerned, the correlation no longer exists. In mice, AmB levels in tissues of the reticuloendothelial system (liver, spleen) in mice, 6 h after an iv injection are of the same order as with L-AmpB33 and AmBisome (Clark et al., 1991; Proffitt et al., 1991), despite the different internalization of the two formulations in J774 cells observed in the present study. It should be remembered that in-vitro studies do not involve sinusoidal endothelium which acts as a filter by only allowing access to the parenchymal cells (via fenestration of approximately 0.1 μm diameter) to small particles, such as sonicated unilamellar vesicles (60 nm) of AmBisome. Furthermore, it is well known that negatively charged, sonicated, unilamellar vesicles are preferentially removed from blood and associated with hepatocytes (Scherphof et al., 1986).

The decreased toxicity of the AmB formulations for J774 and CHO cells, as compared with free AmB, could have two origins. With AmBisome, AmB-liposomes, EGAM 20 and EDAM 20, the reduced internalization is sufficient to explain the lower toxicity. The fact that AmB is internalized as a lipid formulation and not in the free form may also play a role. This may apply for L-AmpB, L-AmpB33 and AmB-cholesteryl sulphate complexes.

As far as the release of AmB from cells is concerned, it is interesting to note that quantitative absorption measurements in the external medium after reincubation in fresh medium revealed that AmB was found in its unbound form, with a characteristic maximum of absorption at 409 nm. This suggests that the AmB formulations are degraded inside the lysosomal compartments of the macrophages, and that the efflux of free drug results from the AmB concentration gradient between lysosomal compartments and the extracellular medium. This kind of efflux process has already been reported for large liposomes (DMPC/DMPG 7:3) encapsulating muramyl dipeptide and human monocytes (Mehta et al., 1982), with large unilamellar vesicles containing labelled lipids and Kupffer cells (Dijkstra et al., 1984), and with pefloxacin and J774 cells (Carlier et al., 1991). The fact that large AmB liposomes, which are rapidly internalized by macrophages, could slowly release free drug outside the cells, could be very useful for the treatment of disseminated fungal disease. In fact it means that in vivo, for some types of liposomes, macrophages could be simultaneously a target and a reservoir for AmB, and so able to deliver the drug gradually to infected tissues, as recently suggested by Mehta et al. (1994). Under these conditions, the threshold of AmB self-association would not be reached in the plasma, and toxicity (at least induction of K+ leakage) would not develop (Bolard et al., 1991).

The fact that the internalization of liposomal AmB varies greatly between different formulations indicates that this 'reservoir' role for macrophages may not be universal for all of these, and that other mechanisms to explain the increases in therapeutic index must exist. This study is of particular interest since in systemic fungal disease it is not
yet known whether the chemotherapeutic effect of the AmB preparations is principally due to the localization of the drug inside macrophages or to the slow release of free AmB in the circulation and tissues.

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References

Liposomal and lipid formulations of amphotericin B. Clinical pharmacokinetics. Clinical 
Pharmacokinetics 23, 279-91.

(1991). Comparative safety, tolerance and pharmacokinetics of amphotericin B lipid complex 
and amphotericin B deoxycholate in healthy male volunteers. Journal of Infectious Diseases 
164, 418-421.

by the polyene antibiotic amphotericin B and deoxycholate. A spin label study. Biochimica 
et Biophysica Acta 981, 207-12.


(1989). Treatment of systemic fungal infections with liposomal amphotericin B. Archives of 
Internal Medicine 149, 2533-6.

amphotericin B by liposomal encapsulation: comparison of normal mice infected with 
Candida albicans. Cancer Drug Delivery 1, 199-205.

B action: accumulation in human monocytes potentiates killing of phagocytosed Candida 

liposome-encapsulated muramyl dipeptide by human peripheral blood monocytes. Journal 
of Reticuloendothelium Society 32, 155-65.

as mechanism for enhanced therapeutic activity of liposomal amphotericin B. Chemotherapy 
40, 256-64.

of liposomes by liver and spleen. Biochimica et Biophysica Acta 761, 142-51.

intraphagocytic Candida albicans. European Journal of Clinical Microbiology and Infectious 
Diseases 9, 738-44.

Pharmacology and toxicology of a liposomal formulation of AmB (AmBisome) in rodents. 

amphotericin B encapsulated in liposomes (AmBisome) in the treatment of invasive fungal 
infections in immunocompromised patients. Journal of Antimicrobial Chemotherapy 28, 
Suppl. B, 73-82.

Single dose pharmacokinetics and tolerance of a cholesteryl sulfate complex of 
amphotericin B administrated to healthy volunteers. Antimicrobial Agents and Chemotherapy 
35, 1029-34.

Uptake and intracellular processing of targeted and non targeted liposomes by rat 
Kupffer cells in vivo and in vitro. Annals of the New York Academy of Sciences 446, 
368-84.

Pilot study of amphotericin B entrapped in sonicated liposomes in cancer patients 

de systèmes colloïdaux dispersibles de lipides amphiphiles sous forme de liposomes 


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