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The structure of colloidal formulations of amphotericin B

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Summary

Fluorescence quenching techniques have been used to identify the location of amphotericin B in liposomes and emulsions. Amphotericin B efficiently quenched the fluorescence of probes located in either the phospholipid bilayer of liposomes or the lecithin emulsifier layer of emulsions, but were without significant effect on the fluorescence of probes in the aqueous surroundings of liposomes or emulsions, or probes in the internal aqueous spaces of liposomes. From these data, and a consideration of the solubility of amphotericin B in solvent systems, it is suggested that amphotericin B in liposomes is present largely in the phospholipid bilayer, and not in the aqueous space or surrounding medium. Additionally, amphotericin B in an emulsion formulation is located in the lecithin emulsifier monolayer and not in the bulk of the oil droplet. These findings are consistent with current views of the mode of action of such formulations.

Introduction

Amphotericin B is the current drug of choice for the treatment of deep-seated mycoses, such as those occurring in transplant or AIDS patients (Medoff et al., 1983). It shows selective toxicity to fungal cells, but significant side-effects to human cells are also observed. These are frequently manifested as central nervous system damage, nephrotoxicity, and general malaise. The mode of action of the polyene antibiotics has been recently reviewed by Bolard (1986).

The normal method of administering amphotericin B, which is insoluble in most parenterally compatible solvents, is as a solubilizate in sodium deoxycholate, which is marketed under the trade name 'Fungizone' (Squibb). Recent work by Juliano and co-workers (Mehta et al., 1984; Hopfer et al., 1984; Juliano et al., 1985; Lopez-Berestein et al., 1985; Juliano et al., 1987) has demonstrated that amphotericin B administered in liposomes has a much reduced toxicity relative to the solubilized formulation. Additionally, we have demonstrated (Davis et al., 1988; Forster et al., 1988) that similar therapeutic advantages can be gained by administering amphotericin B in a soya oil/water emulsion formulation, and that formulations of amphotericin B solubilized in other surfactants such as poloxamers have a toxicity, as measured by erythrocyte lysis, similar to that of the deoxycholate-solubilized material.

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Liposomally entrapped drugs are usually considered to consist of a phospholipid vesicle with the drug contained as an internal aqueous solution. Since amphotericin B is insoluble, or forms aggregates, in aqueous solution, the structure of a liposomal formulation is uncertain. Some possibilities are as follows.

(a) It may precipitate as a finely divided solid in the aqueous space of the liposome.
(b) It may be solubilized in the aqueous space by a solvent sheath of the solvent in which it was added (usually dimethylformamide or dimethylacetamide).
(c) It may be present in the phospholipid bilayer, in a configuration similar to that in which it exerts its antifungal action.

Additionally, amphotericin B in an emulsion formulation may be present in the oil droplet or the interfacial emulsifier layer, or in the aqueous phase surrounded by a cosolvent sheath.

Amphotericin B is a heptaene with an intense structured ultraviolet absorption in the range 300–400 nm. As such it would be expected to quench the fluorescence of any nearby fluorescent molecule emitting in this region of the spectrum by a resonant transfer mechanism (Forster, 1959). We have incorporated fluorescent probes into specific regions of two colloidal formulations in order to locate the amphotericin B molecules. In liposomes, salicylic acid has been used as a fluorescent marker in the aqueous phase, and pyrene in the phospholipid phase. In emulsions, pyrene could be localized in either the oil or the phospholipid layer, and so is not a specific probe for a particular region of the droplet; therefore we have used the pyrene-labelled phospholipid β-(pyren-1-yl)decanoyl-γ-palmitoyl-1-α-phosphatidylcholine (PyrenylPC), which is localized together with the lecithin emulsifier layer on the surface of the oil droplet.

Materials and Methods

Materials

Lecithin was intravenous grade Lipoid E80 kindly donated by Lipoid AG. Pyrene, β-(pyren-1-yl)decanoyl-γ-palmitoyl-1-α-phosphatidylcholine and amphotericin B were purchased from Sigma. Soya oil was purchased locally from J. Sainsbury. All solvents were of Analar grade and were purchased from BDH.

Production of colloidal systems containing fluorescent probes

Liposomal amphotericin B containing pyrene in the phospholipid membrane. Lipoid E80 (20 mg) containing pyrene (20 μg) was made by mixing the materials in methanolic solution and evaporating the solvent. This material was then used to make liposomes by the method of Lopez-Berestein and co-workers (1983) containing varying amounts of amphotericin B. The final samples contained 10 μg/ml of phospholipid, 10 ng/ml of pyrene, and 0–1.5 μg/ml of amphotericin B, in Tris buffer (100 μM, pH 7). The exact concentrations of amphotericin B in the samples were determined by mixing the liposomal amphotericin B suspension with an equal volume of dimethyl sulphoxide and determining the absorbance at 412 nm ($\epsilon_{412} = 1.24 \times 10^5$).

Liposomal amphotericin B containing salicylate in the aqueous phase. Liposomal amphotericin B was prepared by the method of Lopez-Berestein and co-workers (1983), with amphotericin B concentrations in the range 0–2.4 μg/ml and a phospholipid concentration of 10 μg/ml. Sodium salicylate (10 ng/ml) and Tris buffer (100 μM, pH 7) were added to the aqueous phase prior to liposome formation in order to label both the internal aqueous space and the surrounding aqueous medium. Buffering is necessary in this case since the fluorescence quantum yield of salicylate is pH dependent, although constant over the pH range 5–8.

Amphotericin B emulsion containing PyrenylPC in the emulsifier interface. PyrenylPC (100 μg) was mixed with Lipoid E80 (100 mg) in methanol solution. The methanol was removed by evaporation and the mixed phospholipids were used in the preparation of amphotericin B soya oil/water emulsion, using the method described by Davis et al. (1988). The emulsions contained 0–400 μg/ml of amphotericin B, and were diluted into Tris buffer (100 μM, pH 7) by a factor of 100 before recording fluorescence spectra.

Amphotericin B emulsion containing salicylate in the aqueous phase. Emulsions containing amphotericin B (0–400 μg/ml) were made as described by Davis et al. (1988), and diluted by a factor of 100 into Tris buffer (100 μM) containing sodium salicylate (10 ng/ml).

All systems were allowed to equilibrate for 24 h prior to measurement of quenching efficiency.

Fluorescence spectra

Corrected fluorescence spectra were recorded on a Perkin-Elmer 3000 spectrofluorimeter, using a 1-cm square quartz cell for liposomal preparations. Emulsion formulations were too turbid for measurement in this configuration due to scattering of the excitation beam, and so were examined in a 0.5 mm capillary cell mounted at 45° to the excitation and emission axes. Excitation and emission slit widths were 5 nm for liposomal samples; for emulsion samples they were reduced to 2.5 nm to reduce scattered light. All samples had an optical density of less than 0.05 at the excitation and emission wavelengths (10 mm path for liposomal samples, 0.5 mm path for emulsion samples).

Salicylate fluorescence was excited at 292 nm and the emission spectra were recorded from 360–560 nm, with the emission maximum at 410 nm being used for the measurement of quenched intensities. Pyrene and PyrenylPC were excited at 315 nm and the emission spectra were recorded from 330 to 600 nm. The emission maximum at 396 nm was used to quantify the fluorescence intensities. In all cases the pyrene concentration was too low for excimer emission to be observed. Scattering background was reduced using a tangent baseline. No changes in the emission bandshape were observed as the quencher concentration was varied in any of the experiments, thus eliminating the possibility of radiative transfer or inner filter effects. Several very weak impurity emission bands were visible in the region 400–600 nm, but did not interfere with the probe fluorescence.

Data were analysed using a simple Stern-Volmer model of the type:

$$I_0/I_Q = 1 + K_{sv} \cdot [Q]$$

where I_Q and I_0 are fluorescence intensities at quencher concentrations of [Q] and zero, respectively, and K_{sv} is the Stern-Volmer quenching constant.

Results

Liposomal formulations

The Stern-Volmer plot for the quenching of pyrene in liposomes by amphotericin B is shown in Fig. 1. The pyrene fluorescence was rapidly quenched with a Stern-Volmer quenching constant of $9.7 \pm 1.0 \times 10^5$ M^{-1}. The fluorescence of salicylate in the aqueous phase (Fig. 2) was not quenched by amphotericin B in the liposomes, and the fluorescence intensity was seen to get slightly stronger with increasing amphotericin B concentration, leading to an apparent Stern-Volmer constant of $K_{sv} = -1.4 \pm 1.0 \times 10^5$ M^{-1}.

Emulsion formulations

The fluorescence quenching of PyrenylPC and salicylate in the emulsion formulation of amphotericin B is shown in Figs. 3 and 4, respectively. PyrenylPC was strongly quenched by amphotericin B, with $K_{sv} = 6.2 \pm 1.0 \times 10^5$ M^{-1}, whereas salicylate fluorescence was only weakly quenched ($K_{sv} = 1.5 \pm 0.5 \times 10^4$ M^{-1}).

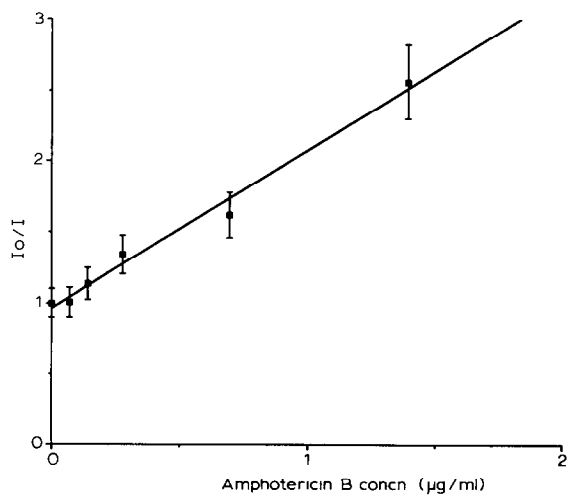

Fig. 1. Stern-Volmer plot for the quenching of pyrene by amphotericin B in liposomes.

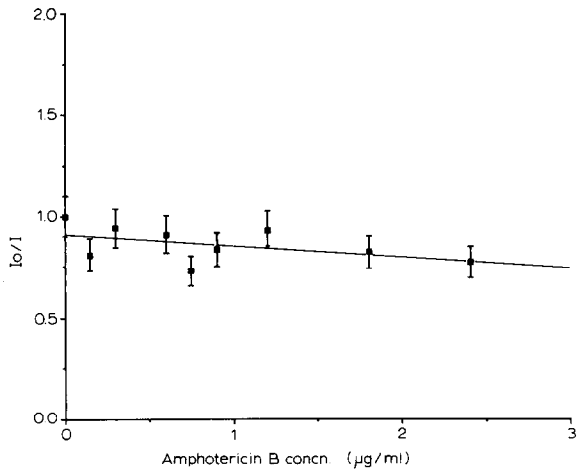

Fig. 2. Stern-Volmer plot for the quenching of salicylate by amphotericin B in liposomes.

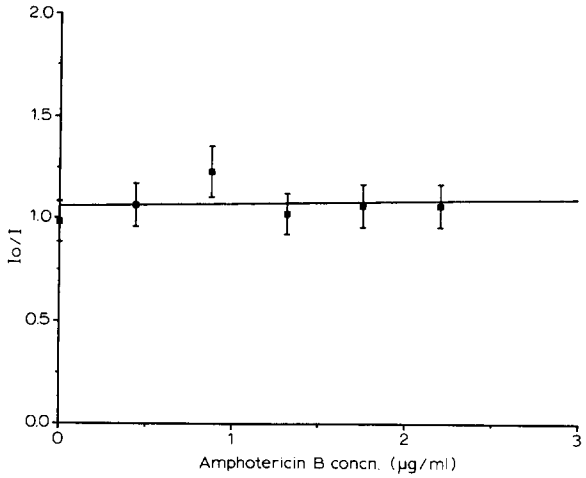

Fig. 4. Stern-Volmer plot for the quenching of salicylate by amphotericin B in an emulsion formulation.

It must also be confirmed that amphotericin B is capable of quenching the fluorescence emission of salicylate in a homogenous medium. This is demonstrated in Figure 5, the Stern-Volmer plot for salicylate quenching by amphotericin B in methanol. The emission was strongly quenched, with $K_{sv} = 5.5 \pm 0.2 \times 10^5\ M^{-1}$.

Discussion

The majority of dynamic quenching processes in solution occur via long-range induced dipole interactions (resonant transfer or Forster-type transfer; Forster, 1959). In order for this process to be efficient, the quencher (acceptor) absorption spectrum must efficiently overlap the emission (donor) spectrum of the species being quenched. The spectra of the compounds studied here are shown in Fig. 6; it can be seen that the intense absorption spectrum of amphotericin B overlaps the emission from the pyrene and salicylate probes. Thus it is highly likely that resonant transfer is the quenching mechanism operating in these systems. Emission from the acceptor can normally be de-

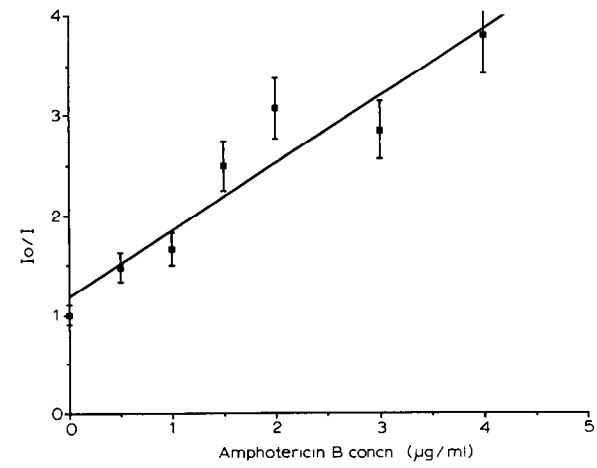

Fig. 3. Stern-Volmer plot for the quenching of PyrenylPC by amphotericin B in a soya-oil/water emulsion.

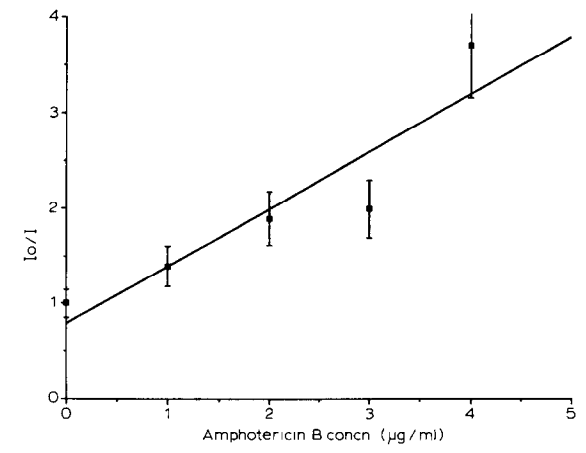

Fig. 5. Stern-Volmer plot for the quenching of salicylate by amphotericin B in methanol.

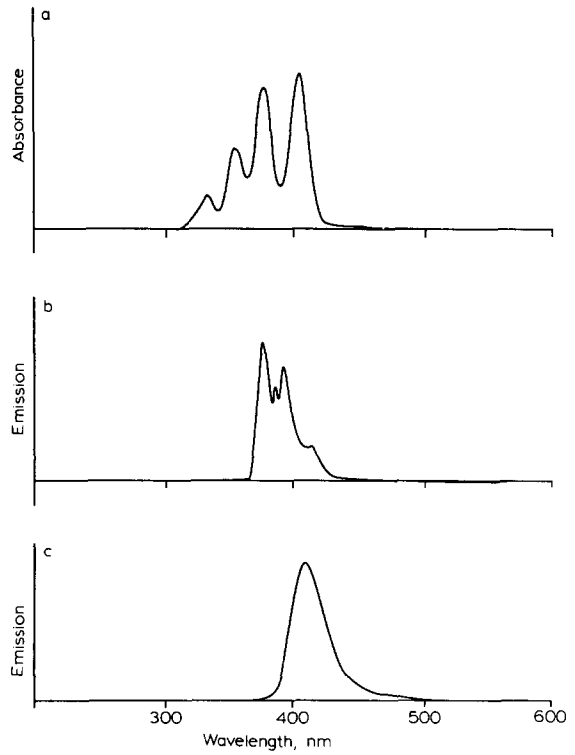

Fig. 6. Spectra of probes and quencher; (a) amphotericin B in methanol (absorption); (b) pyrene in lipid (emission); (c) salicylate in water at pH 7 (emission).

tected if it has a sufficiently high fluorescence quantum yield; however, amphotericin B is non-fluorescent and so no sensitized fluorescence was observed in these experiments. Reports of amphotericin B fluorescence have been shown to be due to impurities (Petersen and Henshaw, 1981).

Quenching processes in general are highly dependent on emitter–quencher separation, and so can be used to infer proximity of the two species. Resonant transfer is no exception, the transfer rate k_q being given by:

$$k_q = (1/\tau) \cdot (R_0/R)^6$$

where τ is the donor lifetime, R the average donor–acceptor separation, and R_0 the critical transfer distance, which can be evaluated by measurement or from spectroscopic parameters (Brand and Witholt, 1967). Generally it lies in the range 2–4 nm, a sufficiently large distance to be described as 'long-range' energy transfer, and which leads to useful quenching efficiencies at relatively low quencher concentrations.

Consequently the observation that pyrene and PyrenylPC were strongly quenched in both liposomes and emulsions suggests that amphotericin B was present in the same phase as the phospholipids, and the absence of significant quenching of the salicylate suggests that amphotericin B was not present in this phase.

The position of the probes in the system is relatively unambiguous. A considerable number of studies (see e.g. Galla and Hartmann, 1980) have demonstrated that pyrene and pyrene-labelled hydrophobic molecules partition spontaneously into phospholipids, and this has been a major tool in membrane biophysics. Consequently we can assume that the pyrene incorporated into liposomal amphotericin B was present only in the phospholipid bilayers and not in the aqueous space or surrounding medium. The strong quenching indicates that amphotericin B was also present in the phospholipid bilayer.

Pyrene is soluble in both phospholipid bilayers and triglycerides, and as such would not be capable of distinguishing between these phases in an emulsion. Consequently PyrenylPC, which behaves similarly to the phospholipid emulsifier used, was chosen to examine the interface, as the polar phospholipid headgroup prevents penetration of the molecule into the oil phase; the packing of the emulsifier was also minimally disturbed by this material since it is chemically and physically very similar to phosphatidylcholine. The quenching of its fluorescence by amphotericin B suggests that the antibiotic was located in the interfacial region of the emulsion. In both systems, the freely water-soluble salicylate ion was unquenched or weakly quenched, indicating that the concentration of amphotericin B in these phases was much lower than in the phospholipid phase. It was necessary to confirm that amphotericin B can quench salicylate in an homogeneous system, as shown in Fig. 5.

It could be suggested that amphotericin B was present partly in the oil phase of the emulsion. This is highly unlikely due to the strongly hydroxylic nature of the amphotericin B molecule; in-

deed our preliminary experiments suggest that the solubility of amphotericin B in soya oil is below a detection limit of 10 ng/ml.

The results presented here strongly suggest that amphotericin B in liposomal and emulsion formulations is present in the phospholipid bilayer or monolayer, respectively. There was no evidence for encapsulated amphotericin B solution in the liposomal preparation. This appeared to be present as a phospholipid bilayer containing amphotericin B, presumably in a similar configuration to that whereby it exerts its antifungal action. Similarly the emulsion formulation appeared to present a phospholipid surface with intercalated amphotericin B to the surrounding aqueous phase.

Juliano and co-workers (1987) have recently proposed a model for the action of liposomal amphotericin B which postulates an intermediate aqueous transfer phase, in a similar manner to that in which phospholipid molecules are transferred between phospholipid bilayers. The work presented here is fully consistent with this model; in particular the similarity of the outer surfaces of both emulsion and liposomal materials, consisting of phospholipid with intercalated amphotericin B, would suggest that both formulations display similar therapeutic advantages, a fact which has recently been confirmed in murine studies (Davis et al., 1988). The two formulations have also been shown to be less toxic toward human erythrocytes than solubilized amphotericin B (Forster et al., 1988).

This study illustrates the use of fluorescence techniques to investigate the microstructure of the novel microscopic, microencapsulated and colloidal formulations which are being widely studied as drug delivery systems. It is hoped that studies of this type will be of use in the study of the mode of action of such systems, and will facilitate the discovery of potential new systems for drug delivery.

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