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A kinetic method for measuring functional delivery of amphotericin B by drug delivery systems

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The human toxicity of amphotericin B can be considerably reduced by associating the drug with liposomes of varying lipid compositions. Some lipid compositions are much more effective than others. We show that a simple kinetic fluorescence assay using pyranine as an indirect probe of amphotericin-induced K^+ currents may be used to study different liposomal drug delivery systems *in vitro*. We find that lipid mixtures composed of DMPC/DMPG/amphotericin at a 7:3:1 mole ratio show very slow functional delivery with a preference for ergosterol over cholesterol-containing membrane vesicles. On the other hand, amphotericin delivered from egg phosphatidylcholine liposomes lead to 100-fold increases in K^+ leakage at one-fifth the amphotericin concentration of the 7:3:1 system. The egg phosphatidylcholine system as well as micellar amphotericin also show a slight selectivity towards cholesterol-containing vesicles over ergosterol. These results are consistent with previous clinical and in vitro cellular studies and this technique may prove valuable in screening of other delivery systems.

The polyene antibiotic amphotericin B (AmB) is the most commonly used drug to treat systemic fungal infections, particularly those that occur in patients with lymphomas, leukemias, AIDS, or other immune system compromising diseases [1]. It forms ion channels, causing lethal changes in membrane permeability to many univalent and divalent cations (see Ref. 2 for a comprehensive review). It also leads to cellular oxidative damage [3]. The antibiotic is selectively toxic towards organisms whose membranes contain ergosterol such as fungi [2]. However, the commercial form of AmB, fungizone, also causes acute and chronic side effects in patients taking it. This is especially true when the drug is administered in the high doses and long courses of therapy necessary to fight most fungal infections [1].

To improve the fungal selectivity of AmB, derivatives of the drug as well as new liposomal vehicles for solubilizing and delivering it have been developed. Some of these lipid/AmB preparations have already been tested clinically with good success (see Ref. 4 for a recent review). When AmB was incorporated into a lipid mixture containing dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol (DMPC/DMPG) at a 7:3 molar ratio, it retained its antifungal activity with fewer of the side effects characteristics of Fungizone [5]. Likewise, *in vitro* studies have shown that this and other liposomal AmB delivery systems retain their toxicity toward fungal cells while reducing AmB's activity on cholesterol-containing cells [1,6–8].

This improved selectivity for ergosterol-containing organisms may result from a preferential vehicle-to-target transfer of AmB that relies on the characteristics of both the delivery system and the target cell [1]. We show that such transfer from a DMPC/DMPG/AmB 7:3:1 (mol/mol) lipid mixture (7:3:1 L-AmB) to target small unilamellar vesicles (SUV) is very slow using an *in vitro* fluorescence assay. Our novel functional assay, which uses kinetic measurements of transmembrane K^+ current induced by transfer of AmB to target SUV containing cholesterol or ergosterol, also allows us to examine the question of sterol selectivity.

Egg phosphatidylcholine (EPC) was isolated from fresh eggs using the Singleton procedure [9]. Dimyristoyl PC and dimyristoylphosphatidylglycerol were purchased from Avanti Polar lipids, Alabaster, AL. Purified amphotericin B was a gift from the Squibb Institute for Medical Research (Princeton, NJ). FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was obtained from the Sigma Chemical Company (St. Louis, MO). Laser grade pyranine (1,3,6-pyrenetri-

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sulfonic acid) was purchased from Eastman-Kodak (Rochester, NY).

7:3:1 liposomal amphotericin B mixtures were prepared in a manner similar to Janoff et al. [10]. Care was taken in the following steps to prevent the amphotericin B from being exposed to light and air. The AmB was added at 10 mol% (dry) to the DMPC/DMPG lipid mixture (7:3, molar ratio) in chloroform. The lipids were then dried to a thin film under a stream of argon and placed in a vacuum desiccator for 18–22 h to remove the solvent. The mixture was suspended in 210 mM sucrose, 25 mM Mops buffer at pH 7.2 and sonicated briefly under argon (< 5 min). The total lipid concentration following sonication was 45 mM, while the AmB concentration was 1.8 mM. The mixture was then diluted, with sucrose buffer, to lipid and AmB concentrations of 1.35 mM and 54 μM, respectively. Following the dilution by the stopped-flow syringes, the final concentrations were 1.13 mM for the lipids and 45 μM for the AmB. Lipid and AmB concentrations were determined as in Ref. 11.

Egg PC delivery SUV (PC L-AmB) were prepared using the procedure detailed in Ref. 12; 100 mg/ml PC was sonicated in 100 mM K_2SO_4, 25 mM Mops (pH 7.20) to avoid extensive sonic destruction of sucrose. Following their preparation, the PC SUV were exchanged with the osmolar sucrose buffer by Sephadex G-25 gel filtration and diluted to a concentration of 0.39 mM with 210 mM sucrose, 25 mM Mops (ph 7.20). AmB was added to the diluted sample at 10 μM from a DMSO stock, and equilibrated with the PC SUV for at least 30 min before the stopped-flow runs. Following the dilution by the stopped-flow syringes, the final concentrations were 0.32 mM for the PC and 8.0 μM for the AmB.

The target SUV were prepared using the procedure detailed in Ref. 12; the ergosterol and cholesterol were incorporated at 10 mol% into their respective lipid-chloroform solutions prior to drying and sonication. SUV used for examining K^+ permeability were sonicated in 100 mM K_2SO_4, 25 mM Mops, 2.2 mM pyranine at pH 7.20. The external pyranine was removed by gel filtration against the K_2SO_4 buffer above. The final total lipid concentration of the target SUV were 4.3 mM. FCCP was added to all target liposomes at a concentration of 10 μM.

For measurements of K^+ permeability, gradients of K_2SO_4 were created across the target SUV by osmolar sucrose (210 mM sucrose, 25 mM Mops at pH 7.20 at 25° C ± 1° C) dilution with variable ratio syringes (1 : 5.7 dilution) in an OLIS (Jefferson, GA) converted Durrum D-110 stopped-flow spectrophotometer in the fluorescence mode (excitation wavelength = 450 nm). The AmB drug delivery vehicles, AmB/DMSO micelles (from DMSO), 7:3:1 L-AmB or PC L-AMB, were introduced in the sucrose buffer so that the chemical potential was created simultaneously with the introduction of the drug. More detailed procedures about the procedure and measurements of initial ion currents and permeability coefficient are given in Ref. 12.

Our assay measured pH changes inside the SUV that result from the AmB induced K^+ currents. With the incorporation of the protonophore FCCP into the SUV bilayers, H^+ can equilibrate rapidly across the membrane. Hence, the imposed K^+ gradients coupled with the ionophoric action of AmB cause a H^+ for K^+ exchange that is limited by the rat of induced K^+ efflux. The pyranine molecules entrapped in the target SUV provide a sensitive fluorescence assay for detecting interior vesicular pH changes. Pyranine displays a linear change in fluorescence intensity with changing pH (between pH 6.4 and pH 7.8), decreasing when protonated and increasing when deprotonated. The initial H^+ ion current density, $I_o(H^+)$, can be measured from the initial rate of change of pH assuming a linear response of pyranine fluorescence in this pH region [12]. The initial pH rate of change is extrapolated to $t = 0$ and hence the membrane potential, $\psi = 0$. This allows us to use the approximation $I_0(H^+) \cong I_0(K^+)$ to determine initial K^+ currents. Permeability coefficients (P_0) for K^+ may also be determined from initial currents by taking into account the concentration gradient across the vesicles assuming a simple diffusion mechanism [12].

Figs. 1 A and B shows the result of 7:3:1 L-AmB

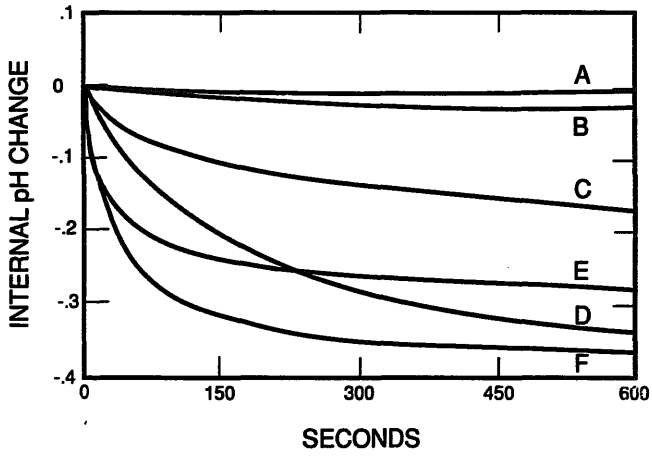

Fig. 1. Liposomal and micellar delivery of AmB to sterol-containing target SUV. K^+ permeability is measured by H^+/K^+ exchange and the pyranine fluorescence changes converted to pH. The baseline permeability of the target vesicles has been subtracted. Over ten minutes, the 7:3:1 L-AmB delivery vehicle (45 μM AmB) reproducibly induced more K^+ loss over time on the SUV with ergosterol (B) than on those with cholesterol (A). The PC-AmB liposomal delivery system (8 μM AmB) showed significantly greater functional delivery of the drug, to target SUV with 10 mol% sterol (ergosterol, C; cholesterol, D) than 45 μM of the drug in its 7:3:1 L-AmB form. 45 μM of 'free' (micellar) AmB causes even greater K^+ leakage in EPC target SUV (ergosterol, E; cholesterol, F). The latter two systems exhibit a net cholesterol selectivity.

(45 μM AmB) delivery to 10% cholesterol (target PC-chol) and ergosterol (target PC-erg) containing SUV with K_2SO_4 gradients imposed on them. Here, we measured only induced K^+ permeability, since AmB does not increase SO_4^{2-} permeability [2,12]. With the baseline current subtracted, the traces for 7:3:1 L-AmB delivery to target PC-chol SUV show small, reproducible decreases in internal pH, with slightly greater K^+ permeability induced on target PC-erg SUV. The induced initial K^+ ion currents, I_0, were $7.7 \cdot 10^{-9}$ amp/cm^2 for target PC-chol SUV and $14 \cdot 10^{-9}$ amp/cm^2 for the target PC-erg SUV. These small currents correspond to induced K^+ permeability coefficients, P_{K^+}, of only 10^{-12}–10^{-13} cm/s.

PC L-AmB at a much lower AmB concentration of 8 μM was delivered to target PC-chol SUV and target PC-Erg SUV (Figs. 1 C and D). It showed much greater activity on the target SUV than 7:3:1 L-AmB as judged by the initial K^+ ion current and pH change over the time course of the experiments (I_0 (target PC-erg) = $3.7 \cdot 10^{-7}$ amp/cm^2; I_0 (target PC-chol) = $6.5 \cdot 10^{-7}$ amp/cm^2). The selectivity has also reversed in this system with target PC-chol SUV becoming slightly more sensitive than target PC-erg SUV. The AmB delivered from this PC L-AmB system was almost as effective as the DMSO micellar form of AmB (at 45 μM), in terms of total induced K^+ loss, although the initial K^+ current was lower. The rapid drug delivery from the PC L-AmB may be due to the fact that AmB is associated with the delivery liposomes largely in a relatively weakly bound monomeric state [11]. Egg PC L-AmB systems have previously been shown to promote high concentrations of AmB in solution as compared to saturated dipalmitoyl PC liposomes [13]. These solution concentrations are correlated with the anticellular effects of AmB [13]. Not surprisingly, liposomal AmB vehicles made from unsaturated lipids such as egg PC are least effective in reducing cytotoxicity in tissue cultures [8].

Above a concentration of about 10^{-7} M, AmB from DMSO or fungizone suspended in an aqueous solution is present in a micellar aggregate form [14–16]. The micellar form of AmB from a DMSO solution, at 45 μM, induced significant permeability on both target vesicles (Figs. 1 E and F) with a greater total loss of K^+ from PC-chol than PC-erg although the initial K^+ currents were identical (target PC-chol and PC-erg, I_0 = $2.3 \cdot 10^{-6}$ amp/cm^2). As a comparison, the K^+ ionophore valinomycin added at 1.5 μM to target PC-erg SUV yielded a similar I_0 for K^+ of $3.7 \cdot 10^{-6}$ amp/cm^2.

One of the interesting results of these experiments is the slightly greater sensitivity of target PC-chol SUV to the action of AmB introduced in the micellar form or from a PC L-AmB delivery vehicle, especially considering conventional wisdom which indicates AmB's greater toxicity against fungi is due to their ergosterol content. Actually, AmB's sterol selectivity between ergosterol and cholesterol vesicles has been shown to be minimal when the drug is added directly from micellar suspensions, especially at higher absolute concentrations (> 5 μM [17,18]). Sterol selectivity toward ergosterol containing liposomes is, however, manifested when AmB is delivered by vesicles made from gel phase saturated lipids [18]. The high concentrations of 'free' AmB (micellar) promoted by incorporation of AmB into egg PC liposomes is probably responsible for the lack of ergosterol selectivity observed in these experiments [13].

Another potential criticism of this method of assessing AmB drug delivery is that SUV are not a very realistic model of biological membranes due to their high radius of curvature. It is certainly true that many characteristics of AmB interaction with membranes are dependent on vesicle geometry [19,20]. However, some properties seem to be very similar. For example, the preferential Ca^{2+} permeability enhancement caused by AmB in ergosterol containing large unilamellar vesicles (LUV) versus cholesterol containing LUV [21] has also been observed in SUV (unpublished results). In any case, the technique outlined here should be useful for studies using more realistic LUV or even cell ghost preparations. We have used this method already to assess AmB-induced K^+ vs. Cl^- permeability in LUV made by reverse evaporation (Hartsel et al. 1990). Drug delivery studies are currently underway in this laboratory using more uniform extruded LUV as an alternative to SUV models.

Our results show that functional transfer of AmB from 7:3:1 L-AmB to target SUV occurs very slowly *in vitro*. PC L-AmB delivery SUV showed a considerably faster rate of transfer to the target SUV than 7:3:1 L-AmB, demonstrating that the presence of lipid alone may not exert a significant protective or selectivity effect. Hydrated 7:3:1 liposomal mixtures may form stable ribbon-like nonliposomal aggregates, distinct from SUV or large unilamellar vesicles, which may reduce the equilibrium concentration of aqueous AmB to which the target cell is exposed [10,22]. This is an important consideration in clinical use because it controls the amount of free AmB the host cells are exposed to at any given time. Most significantly, the 7:3:1 L-AmB was the only preparation showing ergosterol > cholesterol selectivity in this assay. This fits in nicely with clinical and experimental trials showing lower toxicity toward cholesterol-containing organisms. The agreement of these results with cell, animal and clinical studies suggests that this method may offer a rapid, systematic approach to screening new drug delivery systems for membrane-active drugs *in vitro*.

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References

1 Juliano, R.L., Danoud, S., Krause, H.J. and Grant, C.W. (1987) Ann. N.Y. Acad. Sci. 507, 89–103.
2 Bolard, J. (1986) Biochim. Biophys. Acta 864, 257–304.
3 Brajtburg, J., Powderly, W.G., Kobayashi, G.S. and Medoff, G. (1990) Antimicrob. Agents Chemother. 34, 183–188.
4 Brajtburg, J., Powderly, W.G., Kobayashi, G.S. and Medoff, G. (1990) Antimicrob. Agents Chemother. 34, 381–383.
5 Lopez-Berestein, G., Fainstein, V., Hopfer, R., Mehta, K., Sullivan, M.P., Keating, M., Rosenbaum, M.G., Mehta, R., Luna, M., Hersh, E.M., Reuben, J., Juliano, R.L. and Bodey, G.P. (1985) J. Infect. Dis. 151, 704–710.
6 Hopfer, R.L., Mills, K., Mehta, K., Lopez-Berestein, G., Fainstein, V. and Juliano, R.L. (1984) Antimicrob. Agents Chemother. 25, 387–389.
7 Jullien, S., Contrepois, A., Sligh, J.E., Domart, Y., Yeni, P., Brajtburg, J., Medoff, G. and Bolard, J. (1989) Antimicrob. Agents Chemother, 33, 345–349.
8 Szoka, F.C., Milholland, D. and Barza, M. (1987) Antimicrob. Agents Chemother. 31, 421–429.
9 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53–56.
10 Janoff, A.S., Boni, L.T., Poescu, M.C., Minchey, S.R., Cullis, P.R., Madden, T.D., Taraschi, T., Gruner, S.M., Shyamsunder, E., Tate, M.W., Mendelsohn, R. and Bonner. D. (1988) Proc. Natl. Acad. Sci. USA 85, 6122–6126.
11 Hartsel, S.C., Perkins, W.R., Mc Garvey, G.J. and Cafiso, D.S. (1988) Biochemistry 27, 2656–2660.
12 Whyte, B.S., Peterson, R.P. and Hartsel, S.C. (1989) Biochem. Biophys. Res. Commun. 164, 609–614.
13 Jullien, S., Brajtburg, J. and Bolard, J. (1990) Biochim. Biophys. Acta 1020, 39–45.
14 Bolard, J., Seigneuret, M. and Boudet, G. (1980) Biochim. Biophys. Acta 599, 280–293.
15 Ernst, C., Grange, J., Rinnert, H., Dupont, G. and Lematre, J. (1981) Biopolymers 20, 1575–1588.
16 Rinnert, H., Thirion, C., Dupont, G. and Lematre, J. (1977) Biopolymers 16, 2419–2427.
17 Vertut-Croquin, A., Bolard, J., Chabbert, M. and Gary-Bobo, C. (1983) Biochemistry 22, 2939–2944.
18 Vertut-Croquin, A., Bolard, J. and Gary-Bobo, C.M. (1984) Biochem. Biophys. Res. Commun. 125, 360–366.
19 Hartsel, S.C., Benz, S.K., Peterson, R.P. and Whyte, B.S. (1991) Biochemistry 30, 77–82.
20 Milhaud, J., Hartmann, M.-A. and Bolard, J. (1989) Biochimie 71, 49–56.
21 Ramos, H., De Murciano, A., Cohen, B.E. and Bolard, J. (1989) Biochim. Biophys. Acta 982, 303–306.
22 Juliano, R.L., Grant, C.W.M., Barber, K.R. and Kalp, M. (1987) Mol. Pharmacol. 31, 1–11.