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Concentration dependent dual effect of the monolauryl ester of sucrose on the antifungal activity and absorption spectra of amphotericin B (Fungizone)

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A mild detergent, the monolauryl ester of sucrose (LS), at concentrations which ranged from 0.008 to 0.03%, enhanced amphotericin B (AmB) toxicity against *Saccharomyces cerevisiae* and *Cryptococcus neoformans* cells. At higher concentrations, 0.06 to 2.5%, LS inhibited AmB effects on these two fungi. We analyzed changes in the absorption spectrum of AmB induced by LS at these two concentration ranges by comparing ratios (*R* values) of AmB absorbance at 409 nm, the wavelength characteristic of non-aggregated (monomeric) AmB, to absorbance at 328 nm, the wavelength characteristic of aggregated AmB. Low concentrations of LS caused a decrease in *R*, whereas the higher LS concentrations increased *R*. Therefore, LS had concentration-dependent dual effects on the antifungal activity of AmB which correlated with shifts in the physical states of AmB. The concentration range of LS required to inhibit the antifungal effects of AmB was about 1000-fold greater than the previously reported concentrations required to inhibit AmB toxicity to mammalian cells (Gruda, I., Gauthier, E., Elberg, S., Brajtburg, J. and Medoff, G. (1988) Biochem. Biophys. Res. Commun. 154, 954–958). This suggests that LS may be a useful agent to decrease AmB toxicity to host cells without affecting the antifungal effects. Moreover, increase in AmB toxicity induced by low concentrations of LS suggests the possibility that synergistic interaction between fatty acid esters and polyene antibiotics may have therapeutic value.

Introduction

We have recently reported [1] that the toxicity of amphotericin B (AmB) for mouse erythrocytes and cultured mouse fibroblasts, L-929 cells, was decreased by addition of a mild non-ionic detergent, the monolauryl ester of sucrose (LS). This effect of LS was relatively specific because much higher concentrations (1000-fold) were required for a similar inhibition of AmB toxicity to *Candida albicans* cells. Here we measured the effects of a broad concentration range of LS on AmB toxicity to *Saccharomyces cerevisiae* and *Cryptococcus neoformans* cells. We found that the concentration of LS required for the inhibitory effects of LS were comparable to those observed with *C. albicans*. In addition, we found that very low concentrations of LS induced the opposite effect, e.g., they enhanced the antifungal potency of AmB. These concentration-dependent dual effects of LS were also seen on the absorption spectra of AmB. We have tried to elucidate the physical basis of this biphasic action of LS on AmB spectra and its toxicity to cells.

Materials and Methods

Chemicals. Cholesterol and ergosterol were purchased from Sigma, St. Louis, MO. The commercial Fungizone (E.R. Squibb and Sons, Princeton, NJ) was used for AmB. LS (catalog number L-1695) was a generous gift of Mitsubishi-Kasei Food Corporation (Tokyo, Japan). Pinacyanole was purchased from Aldrich Chemical Company (Milwaukee, WI).

Abbreviations: LS, monolauryl ester of sucrose; AmB, amphotericin B.

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Cellular experiments. S. cerevisiae (strain from Hoffman la Roche) and *C. neoformans* (strain 145A) were used to assay the effects of AmB, LS and AmB - LS. Final concentrations of cells were $2.5 \cdot 10^7$ fungal cells per ml of medium. The cell-associated K^+ was measured in a flame photometer [2]; the cell viability was measured by plating cells on Sabouraud agar and counting colonies after 48 h of incubation. Assays were done as described previously [2] with minor modifications [3].

To facilitate comparisons of current data with previous results, concentrations of AmB are expressed as μg/ml in the cellular experiments and as M (mol AmB - 924) in the spectroscopic experiments.

Spectroscopic experiments. Solutions of AmB (as Fungizone), sterols and LS were prepared as described previously [1]. Absorption spectra were recorded with a Varian 634 double beam spectrophotometer.

Results

Effects of LS on the antifungal action of AmB

We compared retention of K^+ and viability of fungal cells exposed to different concentrations of LS alone. AmB alone, and the combined effects of different concentrations of LS and AmB. Fig. 1a shows effects on K^+ retention by *S. cerevisiae* cells. LS alone, at all the concentrations assayed, did not affect the retention of K^+ by *S. cerevisiae* cells. Likewise, 0.06 μg/ml of AmB without LS did not lower the intracellular K^+. AmB concentrations (μg/ml) of 0.12, 0.25, 0.50 and 1.0, in the absence of LS, induced decreases in cellular retention of K^+ to 82, 48, 20 and 5.0% of the control levels, respectively. The addition of 0.0075% LS resulted in slight enhancement of these effects, whereas the maximal enhancement occurred at 0.015% LS. At this concentration, cells retained, 60% (0.06 μg/ml of AmB), 28% (0.12 μg/ml of AmB), 15% (0.25 μg/ml of AmB) and 5% (0.5 μg/ml of AmB) of the control levels of K^+. Stepwise increases in LS concentration (0.03, 0.06 and 0.12%) resulted in less enhancement of AmB action, and levels of 0.25 and 0.5% were inhibitory.

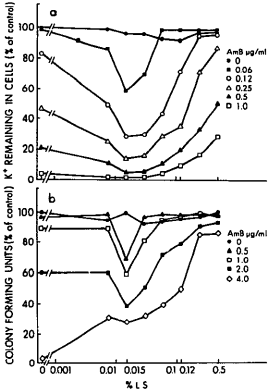

Fig. 1. Effects of LS on AMB-induced decrease in retention of K^+ (a) or viability (b) of *S. cerevisiae* cells. Cells were treated with LS in the absence of AmB and in the presence of concentrations of AmB shown in the figure. Data are from one representative experiment. Similar results were obtained in three other experiments.

The effects of L.S. on AmB induced lethality to *S. cerevisiae* are shown in Fig. 1b. LS alone, at the concentrations assayed, did not affect cell viability. Cells exposed to less than 2.0 μg/ml of AmB alone formed almost as many colonies as control cells, whereas 2.0 μg/ml of AmB decreased *S. cerevisiae* viability to 60% of the control level. When cells were exposed 0.5, 1.0 or 2.0 μg/ml of AmB and to 0.015% of LS, the percentage of viable cells decreased to 70, 60 and 38% of control levels, respectively. The enhancement of AmB lethality by LS was less when 0.03% of LS was used and was not observed at 0.06% of LS. The inhibitory effect of higher LS concentrations occurred as a dose-dependent increase in the ability of AmB-treated cells to form colonies. 4.0 μg/ml of AmB alone killed 95% of the *S. cerevisiae* cells in the absence of LS; all concentrations of LS inhibited the activity of 4.0 μg/ml of AmB and only 15% killing was observed in the presence of 0.5% of LS.

Fig. 2a shows the effects of LS and AmB on retention of K^+ by *C. neoformans*. LS alone, at concentrations of 0.0075–0.12%, decreased K^+ retention from 82 to 75% of control levels. AmB alone, at concentrations of 0.04 or 0.12 μg/ml, decreased K^+ retention to 65 or 20% of control levels. LS at concentrations of 0.015%, added to these concentrations of AmB, increased their effects; K^+ retention was 42% of control levels with 0.04 μg/ml of AmB and 15% with 0.12 μg/ml of AmB. LS concentrations higher than 0.03% reversed the effects of AmB on K^+ retention. Cells treated with 0.04 μg/ml AmB and 0.03% LS or 0.12 μg/ml AmB and 0.12% LS retained about 80% of the control levels of K^+, values comparable to those found in cells treated with these concentrations of LS in the absence of AmB.

The effects of LS on AmB induced lethality to *C. neoformans* cells are shown in Fig. 2b. LS alone, at concentrations of 0.075–0.2%, progressively decreased cell viability from 92 to 80% of control levels. AmB at 0.2 and 0.4 μg/ml, in the absence of LS, decreased cell viability to 78 and 18% of control levels, respectively. In the presence of 0.015% of LS, cell viability decreased to 55% (0.2 μg/ml of AmB) and 5% (0.4 μg/ml of AmB).

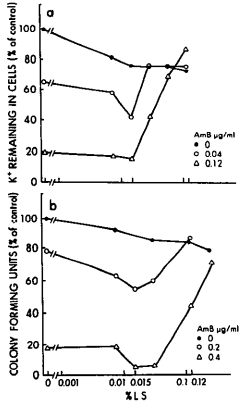

Fig. 2. Effects of LS on AmB-induced decrease in retention of K^+ (a) or viability (b) of *C. neoformans* cells. Cells were treated with LS in the absence of AmB and in the presence of concentrations of AmB shown in the figure. Data are from one representative experiment. Similar results were obtained in three other experiments.

The effects of 0.03% LS were similar to those seen at a concentration of 0.015% LS, whereas concentrations of LS greater than 0.03% inhibited the AmB effects on cell viability. Levels of viability of cells treated with 0.2 µg/ml of AmB and 0.12% LS or 0.4 µg/ml of AmB and 0.25% LS where similar to those caused by LS alone.

From the data presented in Figs. 1 and 2 we calculated the inhibitory potency of LS on the cellular effects of AmB when the latter was used at concentrations which induced decreases in the measured indices to 5–20% of control levels. The calculated molar ratios of LS concentrations required to decrease AmB effects by half to the AmB concentrations used were 17638 (K^+ retention) and 529 (viability) for *S. cerevisiae* and 8808 (K^+ retention) and 6626 (viability) for *C. neoformans* cells. These values are similar to those which have been previously reported for LS inhibitory effects of AmB on *C. albicans* and about a 1000-fold greater than the corresponding values calculated for mouse cells [1].

Therefore although *S. cerevisiae* and *C. neoformans* cells differed in their sensitivity to the toxic effects of LS and AmB (when these compounds were used separately), the pattern of LS – AmB interaction was comparable for these two fungi. Moreover, the pattern was the same for both the permeabilizing and lethal action of AmB. AmB was more active in the presence of low LS concentrations (0.015% of LS induced the greatest change) and in a dose-dependent manner less active with further increases in LS concentrations.

Effects of LS on the physical state of AmB

We next determined how the concentration dependent dual effects of LS on AmB toxicity to cells were related to effects on the physical state of AmB as measured by absorption spectra.

The conformational changes to the AmB molecule caused by aggregation or association with other compounds are reflected in changes in absorption spectra [3–5]. The principle absorption band of free monomeric AmB is located at 409 nm. When the antibiotic aggregates, absorption at 409 nm decreases with a simultaneous increase in absorption at the shorter wavelength regions. We have recently demonstrated [4] that depending on its concentration and on the dispersing power of the medium, small aggregates with maximum absorbance at 328 nm and larger species with a maximum

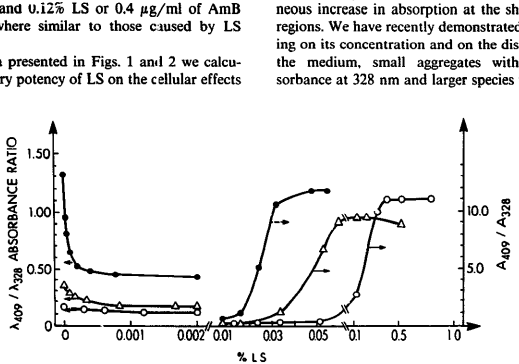

Fig. 3. Effect of LS on ratios of absorbances ($R = A_{409}/A_{328}$) of AmB dispersed in water. ●, $1.5 \cdot 10^{-6}$ M AmB; △, $6.5 \cdot 10^{-6}$ M AmB; ○, $25 \cdot 10^{-6}$ M AmB.

absorbance at 340 nm are formed by AmB. We used the ratio of absorbance at 409 nm to that at 328 nm, $R = A_{409}/A_{328}$, as a measure of the conformational change induced in AmB by its aggregation.

Fig. 3 shows changes of $R = A_{409}/A_{328}$ as function of adding increasing concentrations of LS to three concentrations of AmB ($1.5 \cdot 10^{-6}$ M, $6.5 \cdot 10^{-6}$ M and $25.0 \cdot 10^{-6}$ M) dispersed in water. Depending on the range of LS concentration used, two effects were observed. The progressive decreases in R are depicted on the left side of the figure; the increases in R are presented on the right side of the figure (note the difference in the right and left scales). The decreases in R were first seen for $1.5 \cdot 10^{-6}$ M AmB at 0.00005% LS for $6.5 \cdot 10^{-6}$ M AmB at 0.00008% LS and for $25 \cdot 10^{-6}$ M AmB at 0.0003% LS. The decrease in R was maximal for all solutions of AmB at about the same concentrations of LS (0.002%). In the range of LS concentrations of 0.002–0.01% (not shown in the figure), the decreases leveled off. At concentration of LS higher than 0.01%, the second effect, increase in R, occurred. The progressive increase in R depended on the AmB concentration. R_{max}, characteristic of monomeric AmB (close to 10), was reached at the lowest LS concentration (0.055%) for the most dilute solution of AmB ($1.5 \cdot 10^{-6}$ M); at higher concentrations of LS (0.10%) for the AmB solution of $6.5 \cdot 10^{-6}$ M, and at the highest LS concentration, 0.4%, for the most concentrated AmB solution ($25.0 \cdot 10^{-6}$ M).

We next determined how different concentrations of LS affect the binding of AmB to ergosterol and cholesterol. These experiments were done in a 6.5% propanol/water solution because of the low solubilty of sterols in pure water. For AmB-sterol complexes, the absorption at short wavelengths has a maximum at 348 nm and therefore the ratio of absorbance at 409 nm to that at 348 nm ($R = A_{409}/A_{348}$) was used as a measure of complex formation. Fig. 4 shows the spectral changes induced by different concentrations of LS on the spectrum of AmB alone and in the presence of an equimolar concentration of sterols. In the absence of sterol, LS concentrations up to 0.005% decreased the ratio ($R = A_{409}/A_{348}$) slightly (from $R = 1.4$ to $R = 0.7$). R remained constant with LS concentrations between 0.005 to 0.017%, whereas concentrations higher than 0.017% increased this ratio. At a concentration of 0.14% of LS, R approached the value of 4, characteristic of free monomeric AmB. Thus the relation between LS concentration and its effect on AmB spectra was similar in water/propanol solution and in water. In the absence of LS and in the presence of an equimolar concentration of cholesterol or ergosterol, $R = A_{409}/A_{348}$ was about 0.4. In the presence of increasing concentrations of LS between 0.0002 to 0.017%, R increased slightly, up to about 0.6. In the range 0.017–0.14% LS and in the presence of cholesterol, R increases to 3.5, the value

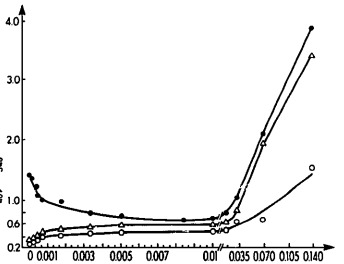

Fig. 4. Effect of LS on ratios of absorbances ($R = A_{409}/A_{348}$) of $6.5 \cdot 10^{-6}$ M AmB dispersed in 6.5% propanol in the absence of sterols (●), in the presence of $6.5 \cdot 10^{-6}$ M cholesterol (△) or $6.5 \cdot 10^{-6}$ M ergosterol (○). Absorbances were measured 45 min after preparation of solutions.

similar to that observed in the absence of sterols. In the presence of ergosterol, the increase in R was much smaller, only up to 1.5. In agreement with previous findings [1], we interpret this result as indicating that, under these conditions, the extent of complexing of AmB with ergosterol was much higher than with cholesterol. The same conclusion could be drawn when AmB without deoxycholate was assayed. In these experiments the concentrated solutions of AmB in dimethyl sulfoxide were added to water or water/propanol mixtures.

Physical state of LS as a determinant of its interaction with AmB

A unique feature of our data was the narrow range of concentrations of LS (0.015–0.03%) in which the LS effects on cellular and spectral properties of AmB changed rapidly. These observations suggested that an abrupt change in the physical state of LS in this concentration range could be responsible for the change in its effects. We therefore determined whether the critical micelle concentration (cmc) of LS occurred at a similar concentration range as the marked shifts in its effects. To determine the cmc of LS, we measured LS-induced changes in the absorption spectrum of the dye Pinacyanole. This dye was used as an indicator because its spectra in a surfactant solution are very diffferent below and above the cmc [6].

Fig. 5 shows a slight increase in the absorbance at two wavelengths of Pinacyanole induced by an increase in LS concentration from 0 to 0.0005% and a plateau in the LS concentration range 0.0005–0.017%. At LS concentrations higher than 0.017%, abrupt changes occurred. These changes indicate that the cmc for LS is

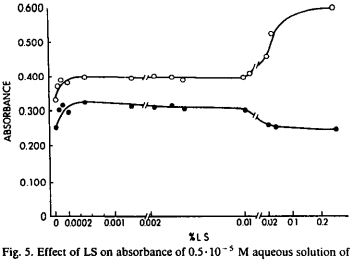

Fig. 5. Effect of LS on absorbance of $0.5 \cdot 10^{-5}$ M aqueous solution of Pinacyanole. Absorbance was measured at 600–611 nm (○) and at 550–567 nm (●).

0.01–0.02%, a value comparable to that determined by others using different methods [7,8]. The similarity between the region of cmc, and the concentrations of LS which resulted in dramatic effects on cellular and spectral properties of AmB implies that all of these phenomena may be related.

Discussion

Two opposite actions of LS on the toxic effects of AmB on fungal cells are presented here: an enhancement and an inhibition. LS is a detergent, and our observations add to the numerous previous reports on the biphasic effects of detergents on absorption and activity of a variety of different drugs. The mechanisms of the duality of action of the detergents may differ with each drug and also with each detergent [9]. We have demonstrated here that the type of LS effect changed when the concentration of LS shifted from below to above the cmc. The changes in LS effects on the antifungal action of AmB were parallel to changes in AmB absorption spectra; the latter reflect the physical state of AmB caused by aggregation or formation of complexes.

At concentrations below the cmc, LS potentiated AmB toxicity to cells and decreased the absorption ratio A_{409}/A_{328} (409 is the peak characteristic of monomeric AmB and 328 is the peak characteristic of aggregate AmB). The simplest explanation of these two observations is that in this concentration range LS existed in the form of aggregates and that complexes were formed between these aggregates and AmB. It is possible that deoxycholate molecules were also associated with these complexes. The concentration of complexes increased progressively with the increase in LS concentration and reached maximum at cmc of LS. At this concentration the maximal potentiation effect of LS on AmB toxicity to fungal cells was observed. This effect was independent of the concentration of AmB used. It is not clear why incorporation of AmB into LS or LS-deoxycholate aggregates potentiates its antifungal activity. The toxic effects of AmB on cells result from AmB binding to sterols incorporated into cell membrane [10,11], and therefore the possibility that LS acted as a promoter of AmB–sterol interactions has to be considered. The analysis of the LS effects on the absorption spectra of AmB-sterol solutions did not, however, indicate any increase in AmB binding to sterol. Hopfer et al. [12] observed an increase in the anticandidal activity of AmB used together with liposomes. These workers raised the possibility that in complexing with liposomes AmB may be concentrated in the outermost layers of the liposomes, thereby increasing the concentration of available drug to the fungal cells. The alternative explanation is that the potentiation of AmB toxicity to fungal cells is related to a facilitated transport of the antibiotic complexed with LS across the polar fungal cell walls. Although the mechanism of potentiation remains unclear, our observations suggest the possibility that synergistic interactions between fatty acid esters and polyene antibiotics may have therapeutic value.

At concentration above the cmc, LS inhibited AmB toxicity to fungal cells and increased the ratio of the absorption peaks A_{409}/A_{328}. We have already shown that monomeric AmB does not form complexes with sterols [4,13]. Here we observed that progressive increases in LS concentrations caused increases in R and decreases in AmB-interactions with ergosterol. Therefore, although we do not know whether the increase in the monomeric form of AmB, as indicated in the increase in absorption at 409 nm, reflects AmB incorporation into micelles and/or the deaggregation of AmB by these micelles, we can infer that the changes induced by LS in the physical state of AmB impaired its binding to sterols and therefore its anticellular activity. Recently it was demonstrated that the anticellular action of AmB prepared with small unilamellar vesicles obtained from phospholipids could be attributed to the activity of AmB remaining unbound to the vesicles [14,15]. It also seems possible that in the experiments presented here, only AmB which did not undergo LS-induced changes formed bonds with sterols or damaged cells. AmB binds, however, more avidly to ergosterol than to cholesterol [11,16]. Moreover, when AmB exists mainly in a monomeric state, traces of the oligomer form of AmB (most probably a dimer) allow a cooperative reaction of AmB with ergosterol to occur which is not the case with cholesterol [4]. Thus, although at sufficiently high concentrations, LS inhibited AmB binding to both ergosterol and cholesterol and damage to both mammalian and fungal cells [1], the partial decrease in the active form of AmB impaired its binding to cholesterol more than its binding to ergosterol and

damage to mammalian cells more than its damage to fungal cells. Probably, a similar mechanism is at least partially involved in the increased selectivity of the toxicity to fungal compared to mammalian cells of AmB used in combination with lipoproteins [17], lipid vesicles [18,19], or other lipid formulations [20]. The attenuation of toxicity to the host cells may allow the administration of increased doses of AmB to animals or to patients with fungal infections. Although the enhanced therapeutic index of liposomal AmB may be due to a combination of factors, this selective decrease in cellular toxicity of AmB is one of them [20].

In summary, the induction by LS of dual changes on AmB spectra indicates a dual effect of LS on the physical state of AmB. The LS-induced changes in the physical state of AmB can be associated with an enhancement or inhibition of AmB antifungal activity. Both of these effects may have a role in improving AmB therapy.

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References

1 Gruda, I., Gauthier, E., Elberg, S., Brajtburg, J. and Medoff, G. (1988) Biochem. Biophys. Res. Commun. 154, 954–958.
2 Brajtburg, J., Kobayashi, D., Kobayashi, G.S. and Medoff, G. (1982) J. Infect. Dis. 146, 138–145.
3 Ernst, G., Grange, J., Rinnert, H., Dupont, G. and Lematre, J. (1981) Biopolymers 20, 1575–1588.
4 Gruda, I. and Dussault, N. (1988) Biochem. Cell. Biol. 66, 177–183.
5 Strauss, G. (1981) Can. J. Spectrosc. 26, 95–102.
6 Mukerjee, P. and Mysels, K.J. (1971) NSRDS-NBS-36, Superintendent of Documents, U.S., Government Printing Office, Washington, DC 20407.
7 Makino, S., Ogimoto, S. and Koga, S. (1983) Agric. Biol. Chem. 47, 319–326.
8 Osipov, L., Snell, F.D. and Hickson, J.L. (1958) J. Am. Oil Chem. Soc. 35, 127–129.
9 Attwood, D. and Florence, A.T. (1982) Surfactant Systems: Their Chemistry, Pharmacy and Biology. Chapman and Hall, London.
10 Medoff, G., Brajtburg, J., Kobayashi, G.S. and Bolard, J. (1983) Annu. Rev. Pharmacol. Toxicol. 23, 303–330.
11 Bolard, J. (1986) Biochim. Biophys. Acta 864, 257–304.
12 Hopfer, R.L., Mills, K., Mehta, R., Lopez-Berestein, G., Fainstein, V. and Juliano, R.L. (1984) Antimicrob. Agents Chemother. 25, 387–389.
13 Gruda, I. and Bolard, J. (1987) Biochem. Cell. Biol. 65, 234–238.
14 Jullien, S., Vertut-Croquin, A., Brajtburg, J. and Bolard, J. (1988) Anal. Biochem. 172, 197–202.
15 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.
16 Gruda, I., Nadeau, P., Brajtburg, J. and Medoff, G. (1980) Biochim. Biophys. Acta 602, 260–268.
17 Brajtburg, J., Elberg, S., Bolard, J., Kobayashi, G.S., Levy, R.A., Ostlund, R.E., Jr., Schlessinger, D. and Medoff, G. (1984) J. Infect. Dis. 149, 986–997.
18 Mehta, R., Lopez-Berestein, G., Hopfer, R., Mills, K. and Juliano, R. (1984) Biochim. Biophys. Acta 770, 230–234.
19 Juliano, R.L., Grant, C., Barner, K.R. and Kalp, M.A. (1986) Mol. Pharmacol. 31, 1–11.
20 Janoff, A.S., Boni, L.T., Popeseu, 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.