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Full Length Research Paper

Modulation of the polyene antibiotic amphotericin B selective toxicity by pH change of the stock solutions

Belkherroubi-Sari L[1], Boucherit Z[1], Chéron M[2], Boucherit K[1], Benyoucef M[1] and Belbraouet S[3]

[1]Laboratoire Antibiotiques Antifongiques : physico-chimie, synthèse et activité biologique, Université Abou Bekr Belkaïd Tlemcen Algérie.

[2]BioMoCeTi, UMR CNRS 7033, Université Pierre et Marie Curie, Paris, France.

[3]Corresponding author École de Nutrition, Université de Moncton, Moncton Canada.

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Stock solutions of the polyene antibiotic amphotericin B (AmB) were prepared at pHs from 4.0 to 5.4 in acetic acid/acetate buffer, at 7.4 in phosphate-buffered saline (PBS), and from 10.0 to 10.8 in carbonate/bicarbonate buffer. Their antifungal activity was tested on *Candida albicans* cells and their toxicity on human erythrocytes. Antifungal activity was observed for all preparations. Surprisingly, the toxicity (K^+ release and hemolysis) drastically decreased for the two preparations at pH 5.4 and 10.8. This effect was attributed, in the first case, to a screening of the positively charged aggregated antibiotic by the negatively charged acetate ions of the buffer, and, in the second case, to a neat modification of the mode of AmB self-association.

Key words: AmB, pH, toxicity, erythrocytes, *C. albicans*.

INTRODUCTION

The polyene antibiotic amphotericin B (AmB) is the drug of choice in the treatment of systemic fungal infections in spite of its high toxicity. A large amount of data has shown that this toxicity is related to its low aqueous solubility. Permeability to K^+ is thought to be the first event following the addition of AmB to cells and therefore an indicator of its activity (Bolard, 1986; Bolard, et al., 1991; Hartsel and Bolard, 1996). Several strategies have been implemented in order to increase the therapeutic index of AmB, e.g. as modification of the drug's chemical structure or modulation of its solubility (Dotis et al., 2006; Wasan et al., 1994; Wong-Beringer et al., 1998). However, these preparations are too expensive for poor or developing countries.

In aqueous solutions, AmB is a mixture of species, that is, soluble monomers, water- soluble self- associated and water-nonsoluble aggregates (Legrand et al., 1992). Studies have shown that the soluble and nonsoluble aggregates of AmB are the most toxic towards animal cells (Cybulska et al., 1995; Gaboriau et al., 1997), although the water -nonsoluble aggregate of AmB is less toxic (Gaboriau et al., 1997). Moreover, the state of aggregation of the AmB molecule can be modulated by the physicochemical environment, e.g. temperature, light, ionic strength and pH (Dupont et al., 1977; Ernst et al., 1978; Hung et al., 1988). In Tris-hydroxymethyl buffer, Dupont et al. (1977) have shown a modification of the state of aggregation of the AmB molecule under the effect of pH. When the pH varied from 7.4 to 10.0, they observed an important increase in the size of AmB water-nonsoluble aggregates.

Based on the above data, we studied the effect of pH modulation of AmB stock solution on its biological activity and on its state of aggregation**.**

MATERIALS AND METHODS

Preparation of the human erythrocyte suspension

Human venous blood (from one healthy donor) was collected in tubes containing heparin. The tubes were centrifuged at 1500 x *g* for 10 min to remove the plasma and buffy coat. Erythrocytes were

*Corresponding author. E-mail: slimane.belbraouet@umoncton.ca

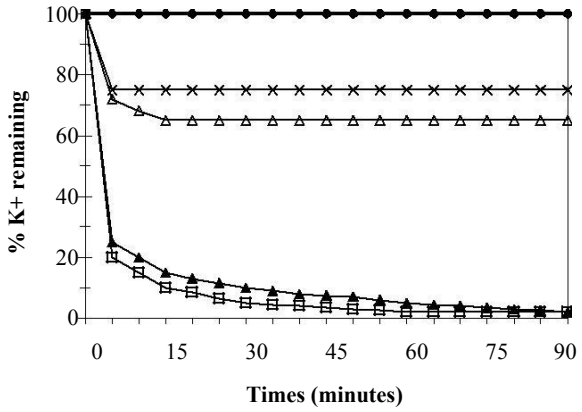

Figure 1. Kinetics of AmB-induced release of K^+ from human erythrocytes, showing the influence of the pH of the stock solutions of AmB (final concentration at 10 µg/ml). (- - Control, - -DMSO, - - pH 4.0, 4.4, 5.0, 7.4 ,10.0, -×- pH 5.4, - - pH 10.8)

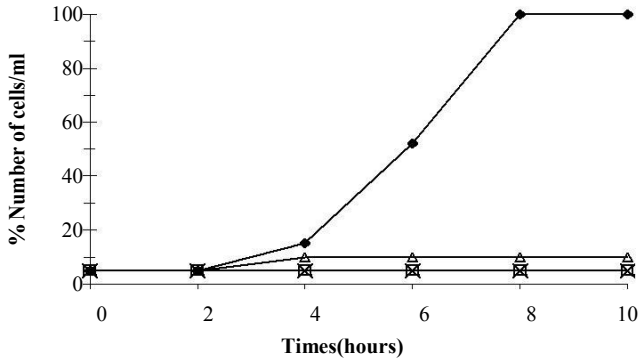

Figure 2. Influence of the pH of the stock solutions of AmB (final concentration at 2 µg/ml) on the growth of *Candida albicans*. (- - Control, - -DMSO, -×- pH 4.0, 4.4, 5.0, 5.4, 7.4, - - pH 10.0, 10.8)

then washed three times with PBS (150 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4; pH 7.4), suspended in PBS at a hematocrit of 40% and used on the same day.

Preparation of the antifungal solutions

Pure AmB (SIGMA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10^{-2} M. From this stock solution, antifungal solutions were prepared at 10^{-4} M in carbonate/bicarbonate buffer (10 mM) of which the pH was adjusted from pH 10.0 to 10.8, and in acetic acid/acetate buffer (0.1 N) of which the pH was adjusted from pH 4.0 to 5.4. These solutions were kept at ambient temperature and out of light for 20 min before they were used.

Measurement of antibiotic-induced K^+ leakage from erythrocytes and haemolysis

Appropriate amounts of antifungal solutions were added to PBS at 37°C at a final concentration of 10 µg/ml. The suspension was immediately mixed by vortexing and incubated for 5 min at the same temperature. Erythrocytes were then added to a final concentration of 4 x 10^3 cells/ml and incubated at the same temperature. For the kinetic measurements, samples were taken at 5, 10, 15, 30, 60 and 90 min. Each sample was rapidly squirted into an ice- cold hemolysis tube containing 2 ml of 150 mM NaCl, 2 mM $MgCl_2$. The suspension was immediately centrifuged at 4000 x *g* for 5 min. K^+ leakage was determined by flame emission at 766 nm (JENWAY/PFP7). Cellular hemolysis was determined by optical density at 548 nm. All experiments were performed three times in duplicate.

Measurement of antifungal activity against yeasts

An inoculum of *C. albicans* was added to Sabouraud broth at 37°C, and the suspension was incubated for 5 min at the same temperature with continuous agitation. The final concentration of yeast was 12 x 10^6 cells/ml. Appropriate amounts of AmB solutions were added to the culture at a final concentration of 2 µg/ml. For kinetic growth measurements, samples were taken at 2, 4, 6, 8 and 10 h. The yeast cells were enumerated using a counting chamber. All experiments were performed three times in duplicate.

Spectroscopic measurement

Circular dichroism (CD) spectra were recorded with a JASCO J-810 dichrograph, thermostatted at ambient temperature. The differential molar dichroic absorption coefficient ($M^{-1}.cm^{-1}$) is . Electronic absorption spectra were recorded with a Cary 219 (Varian) spectrophotometer.

RESULTS

AmB-induced K^+ leakage from erythrocytes

According to the results obtained (Figure 1), the solutions of AmB prepared at pH 4.0, 4.4 and 5.0 in acetic acid/acetate buffer, 7.4 in PBS, and 10.0 in carbonate/bicarbonate buffer, induced massive K^+ leakage from erythrocytes (80 to 90%) after 5 min of incubation. No K^+ leakage was observed after 90 min. Similar results were obtained with AmB solutions prepared in DMSO. However, antifungal solutions prepared at pH 5.4 in acetic acid/acetate buffer, and at pH 10.8 in carbonate/bicarbonate buffer, induced only 20 to 25% of intracellular K^+ leakage after 90 min of incubation. Moreover, at these two pHs, the toxicity of AmB, with a final concentration of 20 µg/ml, towards erythrocytes was largely reduced (by 70%). In contrast, at a concentration of 8 µg/ml, the antifungal solutions prepared in DMSO induced complete lysis of the red blood cells (data not shown). Buffers at each pH lacking AmB did not cause leakage of K^+ from erythrocytes.

AmB-induced inhibition of yeast growth

Figure 2 shows that the antifungal solutions prepared at pH 4.0 to 5.4 in acetic acid/acetate buffer (0.1 N), at pHs

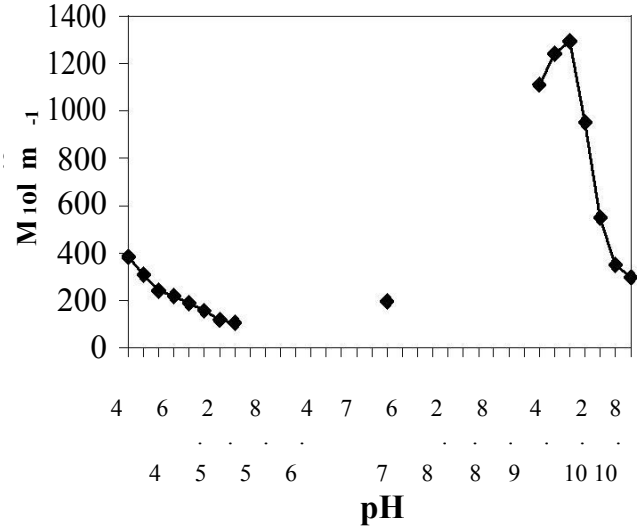

Figure 3. Effect of the pH stock solution of AmB (10^{-4} M) on the CD spectra wavelength at 328 nm for pH<7.4 (acetic acid/acetate buffer) and pH 7.4 (PBS), and between 329 and 337 nm for pH>7.4 (carbonate/bicarbonate buffer).

10.0 and 10.8 in carbonate/bicarbonate buffer (10 mM),and at pH 7.4 in PBS, induced total inhibition of growth of *C. albicans* . Similar results were obtained with solutions prepared in DMSO. Buffers at each pH lacking AmB did not inhibit growth of *C. albicans*.

CD spectra

Under our experimental conditions, the AmB CD spectra are dominated by an excitonic doublet centred around 340 nm. An increase in the pH of the buffer (acetic acid/acetate) from 4.0 to 5.4, resulted in a progressive decrease of this doublet (its intensity is divided by 4), without a change in its wavelength (Figures 3 and 4A). At pH 7.4 in PBS, the doublet intensity was of the same order of magnitude. At pHs 9.5 to 10.0 in carbonate/bicarbonate buffer, the doublet intensity was very high but decreased abruptly at higher pHs (its intensity is divided by 6 at pH 10.5; Figure 3) and its wavelength was shifted from 340 to 350 nm (Figure 4B).

The different AmB species responsible for the CD spectra were separated by centrifugation according to a procedure described by Legrand et al. (1992). Indeed, three species could be distinguished, that is, monomeric AmB (presenting a very weak CD), water-soluble self-associated AmB (presenting a strong dichroic doublet), and water-nonsoluble aggregated AmB (also presenting a strong dichroic doublet). For pHs 4.0 to 5.4, the percentage of monomeric and self- associated water-soluble AmB was low with most of the AmB sedimenting upon centrifugation. In contrast, at pHs between 10.0 and 10.8, AmB was totally soluble (Figure 5).

DISCUSSION

It appears that irrespective of the AmB stock solutions used, the antibiotic at a concentration of 2 g/ml totally inhibited growth of *C. albicans* in Sabouraud medium (pH 5.8). This shows, in particular, that no chemical decomposition occurs at extreme pHs. In contrast, AmB toxic activity, at a concentration of 10 g/ml against erythrocytes in PBS at pH 7.4, depended on the nature of the stock solution preparation. Preparations at pHs 4.0 to 5.0 in acetic acid/acetate buffer, at pH 7.4 in PBS, and at pH 10.0 in carbonate/bicarbonate buffer, induced K^+ leakage and hemolysis. Most interestingly, preparations at pH 5.4 in acetic acid/acetate buffer and at pH 10.8 in carbonate/bicarbonate buffer were not toxic.

How does the spectroscopy data explain these observations? We should first recall that aqueous solutions of AmB contain several forms of AmB:

Monomers, active against *C. albicans*, nonactive against erythrocytes, presenting a weak CD.
Water-soluble self-associated AmB, active against *C. albicans* and erythrocytes, presenting a strong dichroic doublet centred around 340 nm.

Water -nonsoluble aggregated AmB, poorly active against *C. albicans* and erythrocytes, presenting a strong dichroic doublet centred around 340 nm.

Water -nonsoluble aggregated AmB, poorly active against *C. albicans* and erythrocytes, presenting a strong dichroic doublet centred around 320 nm.

At neutral pH, the AmB molecule is zwitterionic due to the presence of one carboxylate group and one protonated amino group. At a lower pH, carboxylate is protonated [pK = 5.5 (Smith and Rawlins, 1973)] and the molecule is positively charged.

It is generally considered (Bolard et al., 1991) that in their self-associated form, AmB molecules are stacked in a helicoidal arrangement. At low pH, the positive charges are located at the surface of the rod formed by this arrangement. Condensation of counter ions (acetate from the buffer) is expected to occur on this polyelectrolyte, according to the popular Manning's model, and we suggest that this screening is at the origin of the decreased toxicity observed at pH 5.4. Consideration of pH dependence of the ion concentration present in the solution should confirm this suggestion by demonstrating the existence of a screening peak at this pH. Indeed, when the pH increased from 4.0 to 7.0, the acetate concentration increases (pK = 4.8) whereas the positive charge of the polyelectrolyte rod decreases. If we deter-mine, as a function of pH, the Debye screening length "r_D" and the decay length " " of the acetate ion concentration profile in the non-linear screening regime, by using the geometrical parameters given in Millié et al. (1999), and assuming a rod radius of curvature "a" = 13 A (according to the

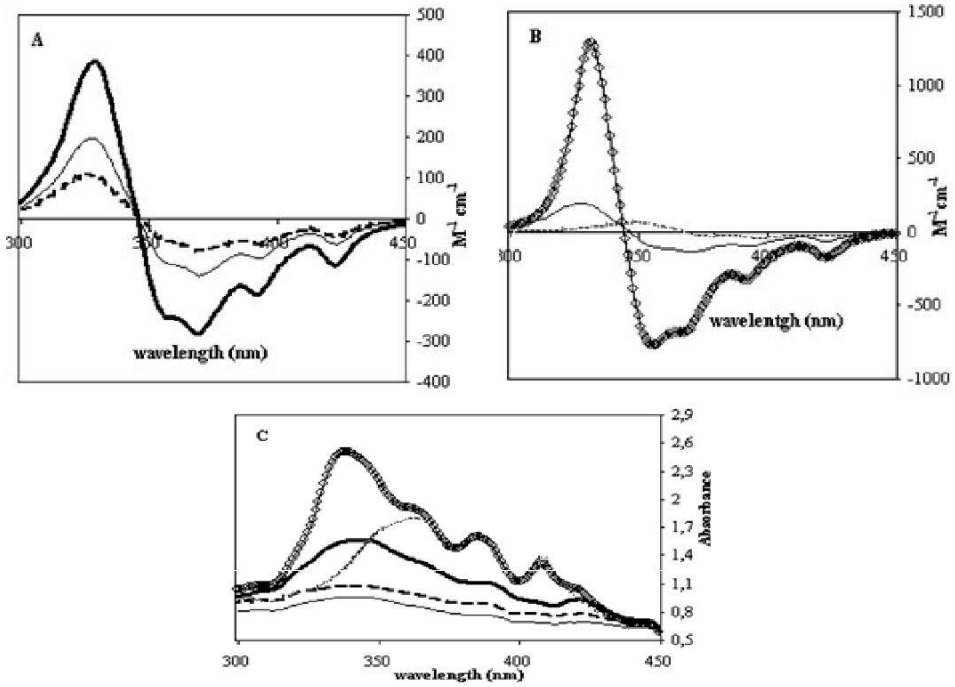

Figure 4. Effect of the modulation of pH for the stock solutions on the state of aggregation of the AmB [(A): CD spectra at pH 4.0, 5.4 and 7.4; (B): CD spectra at pH7.4, 10.0 and 10.8; (C): absorbance spectra]. (pH 4.0, pH5.4, pH7.4, - - pH10.0, ….. pH10.8)

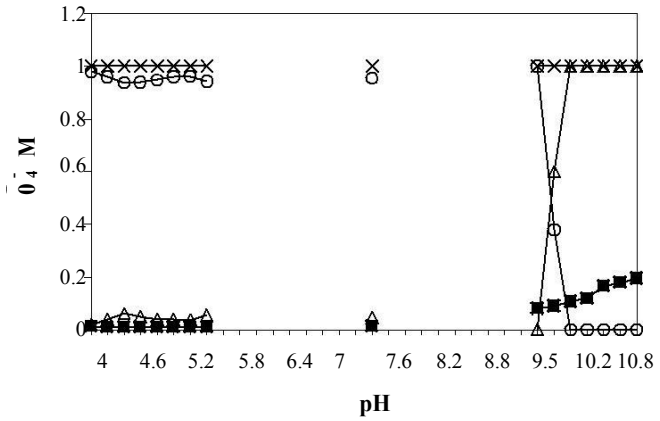

Figure 5. Concentration of the different species of AmB (monomers, water-soluble self-associated and water-nonsoluble aggregates), depending on the pH of the stock solution at 10^{-4} M. (-×- [AmB] total, - - [AmB] monomers+ water-soluble self-associated, - - [AmB] monomers, - - [AmB] water-nonsoluble aggregates).

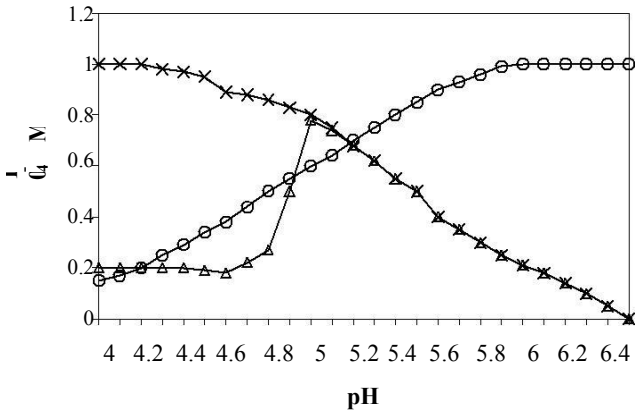

Figure 6. Screening of AmB in the acid acetic/acetate buffer.
(- - charge CH_3COO^-, -×- charge AmB, - - x charge AmB).

the length of bound and double bound given by Ganis et al. (1977), two regimes are obtained that can be interpreted according to Rouzina and Bloomfield (1996):

between pH 4.0 and 5.0, $\lambda < a < r_D$, which corresponds to a non-linear cylindrical regime. Above pH 5.0, $r_D < a < \lambda$,which corresponds to a linear, cylindrical (pseudo-planar) regime. In the first case, the fraction of acetate bound per unit surface charge is $\Theta = 1 - 1/\xi$, ξ being the Manning-Oosawa parameter. It appears that $\Theta = 0.2$ between pH 3.8 and 4.6, and abruptly increased up to 0.98 for pH 5.0. In the second case, the rod surface kept all of its neutralizing charge. However, as the charge of the aggregates decreased due to AmB carboxylate deprotonation, screening also decreased. Figure 6 illus

trates the proportion of neutralizing charge as a function of pH. A sharp maximum was observed at pH 5.0, supporting the existence of a small pH range of protection against AmB toxicity. The discrepancy with the observed pH of maximum protection (pH 5.4) may be explained by the uncertainty of the geometrical model. Our proposal explaining the decreased toxicity by a screening of the self-associated drug could possibly be extended to charged derivatives of AmB, such as AMA (Blanc et al., 2000; Chéron et al., 1988) or MFAME (Szindler-Reichter et al., 2001).

The origin of the strong decrease in toxicity observed at pHs between 10.0 and 10.8 should be found elsewhere, because, at these pHs, AmB is either neutral or negatively charged (the mycosamine NH_2 group's pK is 10.0 (Hung and al., 1988) and should not interact with carbonate anions. However, the charge varies rapidly between 10.0 and 10.8 and indeed a strong modification of the CD spectra were observed. Although almost all AmB remained in the monomeric or water-soluble self-associated forms (Figure 5), the shift in wavelength of the dichroic doublet and decrease in its strong amplitude (Figure 4B) indicated that the mode of self-association changed.

In conclusion, the reduction in the toxicity of AmB towards the red blood cells observed at pH 5.4 is due to antibiotic screening by the acetate ions of the buffer, without reorganization of AmB self- association. However, above pH 10.0, it is necessary to allot the reduction in toxicity to a consecutive reorganization of mycosamine amine deprotonation. We are currently investigating whether the decrease in toxicity of these two preparations observed *in vitro* can be extended to *in vivo* studies.

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