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Effect of the ionic environment on the improvement of the therapeutic index of the Amphotericin B

K.Boucherit[2], Z.Boucherit-Atmani[2], L.Belkherroubi-Sari[2], S.Belbraouet[1]*
[1]Ecole de Nutrition, Universite de Moncton, Moncton, (CANADA)
[2]Laboratoire Antibiotiques Antifongiques : physico-chimie, synthese et activite biologique, Universite, Abou Bekr Belkaid Tlemcen, (ALGERIE)
E-mail : slimane.belbraouet@umoncton.ca
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ABSTRACT

Various salt classes (chlorides: KCl, NaCl, $MgCl_2$, $CaCl_2$; sulfates: Na_2SO_4, $MgSO_4$, Na_3SO_4; carbonates: K_2CO_3, $LiCO_3$, $KHCO_3$) were added to the reactional medium in order to evaluate their effect on the selective activity of amphotericin B (AmB) against the erythrocytes and yeasts (*Candida albicans*). Only $MgCl_2$, used with a final concentration of 10^{-2} M, protects the human red blood cell from the toxic effect of AmB; the erythrocytes lose only 30% of their K^+. This same salt increases the effectiveness of the AmB against *C albicans* by 10%. $MgSO_4$ reduces the toxicity of AmB against the red blood cells by 10%.

KEYWORDS

Amphotericine B;
Chlorides;
Sulfates;
Carbonates;
Toxicity.

INTRODUCTION

Invasive mycoses are associated with high mortality due to the time of the diagnosis and the ground on which they occur. The appearance of resistance to antifungal therapy in certain microorganisms and the inefficiency of an antifungal therapy to protect the organism against invasion from certain pathogens constitute a real problem in public health. Over the past decade, there has only been one antifungal therapy developed.

Despite the significant number of side effects, amphotericin B (AmB), which has been used for more than 40 years, still remains to be the best polyenic antifungal drug in major fungi infections, because of its very broad spectrum, its intense fungicidal effect, and the appearance of relatively few resistance cases[12,21]. The provision of the azolated derivatives, which, contrary to the AmB, are inhibitors of the growth of the fungi cells[13] and are better tolerated, raised great hopes. Unfortunately, there appeared to be cases of increasingly frequent resistances[1,22,24].

To accurately determine the cause of the systemic mycoses, an understanding of the mechanism of action of the amphotericin B, together with its mechanism of toxicity, both based on an interaction of antifungal with membrane sterols, is essential.

The initial step of effectiveness or toxicity consists of an interaction with sterols of the plasmatic membranes, in which this antifungal shapes the pores. This phenomenon involves an escape of the cytoplasmic components, in particular, the potassium ions (K^+). These pores result from the aggregation of several molecules of AmB inside the lipidic double-layered cytoplasmic membrane, so as to form a kind of hollow roll by which essential ions and cellular components can escape[17].

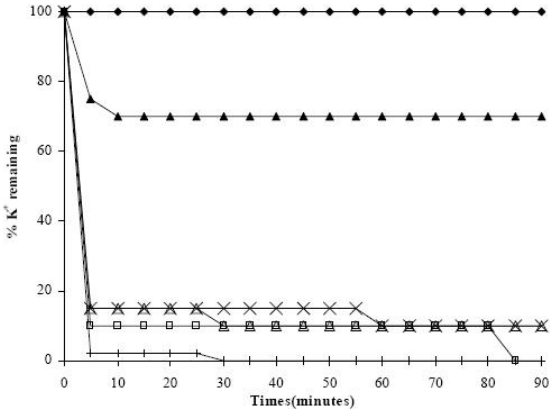

Figure 1 : Kinetics of AmB-induced release of K^+ from human erythrocytes, showing the influence of 10^{-2}M of chlorates (final concentration of AmB at 10^{-5}M). Cells suspensions at 4×10^3 cells/mL. Incubation at 37°C and pH 7.4. (-◆- Control, -□-DMSO, -▲-$MgCl_2$, -×- NaCl, -+- KCl, --△-- $CaCl_2$)

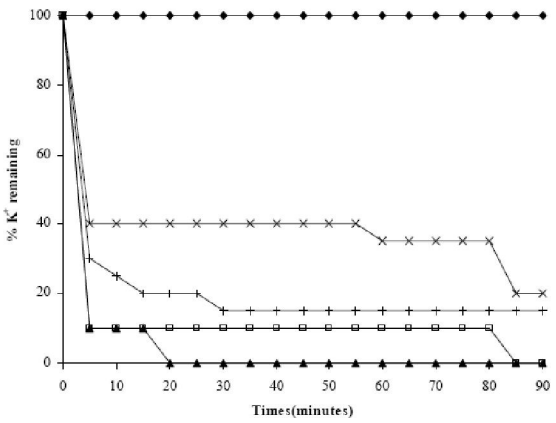

Figure 2 : Kinetics of AmB-induced release of K^+ from human erythrocytes, showing the influence of 10^{-2}M of carbonates (final concentration of AmB at 10^{-5}M). Cells suspensions at 4×10^3 cells/mL. Incubation at 37°C and pH 7.4. (-◆- Control, -□-DMSO, -▲-K_2CO_3 -×- $LiCO_3$, -+- $KHCO_3$)

Moreover, the AmB is insoluble in water[2]. Indeed, in aqueous solution, the antifungal drug was left again schematically in three states[7]: an aggregate form responsible for the toxicity, an oligomeric form (essentially dimeric) endowed with less toxicity, and a monomeric form that is not very toxic, which is responsible for the antifungal activity. Balance between these three forms is not fixed. It varies according to the physicochemical environment of the molecule[15,16]. Then, we understand the importance of the choice of the support of solubilization in order to reduce the proportion of the toxic forms of AmB.

In addition, it is known that the divalent metal ions affect the interaction between antifungal polygenic and the cells. Indeed, previous research has shown that magnesium and calcium disturb the effect of nystatin and AmB on erythrocytes[10] and on *Saccharomyces cerevisiae*[3,11,19].

It appears that salts, in the presence of AmB, play a significant role in the activity of this drug. According to the Hofmeister theory, salts can modify the physicochemical properties of water: Ions known as 'kosmotropic' increase solubility in water, whereas ions known as "chaotropic" reduce solubility[4,25]. The kosmotropic anions, such as sulfate, citrate, or phosphate, reduce the solubility of AmB in aqueous medium, thus valorizing the oligomeric forms, whereas the chaotropic anions, such as thiocyanate and trichloracetate, completely solubilize AmB and its derivatives[24]. Moreover, an increase of ionic force decreases the concentration of the monomers of amphotericin B 3-dimethylaminopropylamid[6] and of *N*-methyl-*N*-fructosyl amphotericin B methyl-ester (MFAME)[23].

In this study, we aim to evaluate the impact of various salts on the selective toxicity of AmB. We studied the effect of carbonates, chlorides, and sulfates on the selective toxicity of the AmB, as well as the phenomenon of 'dormancy' of *Candida albicans* cells under the effect of the AmB complexed with the monovalent and divalent ions, to explain the therapeutic failures related to this drug.

MATERIALS AND METHODS

Biological materials

For the tests of selective toxicity of AmB, we used human red blood cells (universal model of animal cells) coming from a healthy single donor (not presenting any apparent pathology). In addition, we used the yeast *Candida albicans* ATCC 10231 American Type Culture Collection (Rockville, Mandelevium, USA) coming from the Pasteur Institute of Paris. This stock is maintained by successive road repairs on gelose YM preserved at +4°C.

Methods

(1) Preparation of the human erythrocyte suspension

Human venous blood collected (from one healthy

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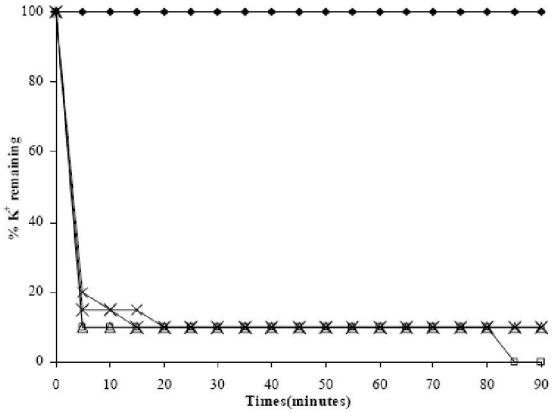

Figure 3 : Kinetics of AmB-induced release of K^+ from human erythrocytes, showing the influence of 10^{-2}M of sulfates (final concentration of AmB at 10^{-5}M). Cells suspensions at 4×10^3 cells/mL. Incubation at 37°C and pH 7.4. (-◆- Control, -□- DMSO, -△-K_2SO_4, -×- $MgSO_4$, -+-Na_2SO_4)

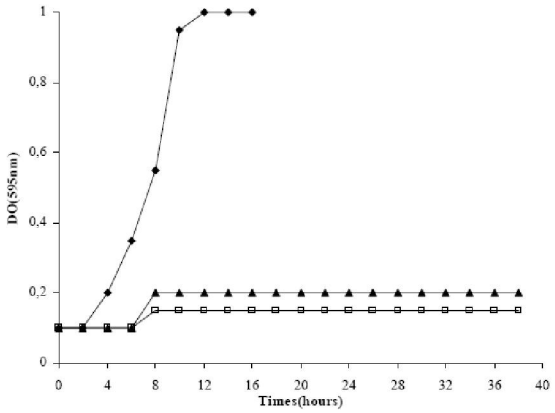

Figure 4 : Influence of 10^{-2}M of carbonates on the growth of *Candida albicans*. Inoculums of 2×10^6 cells/mL. Incubation at 30°C in continuous agitation. (-◆- Control, -▲-K_2CO_3, $LiCO_3$, and $KHCO_3$ at pH 8.5, -□-K_2CO_3, $LiCO_3$, and $KHCO_3$ at pH 6.2)

donor) in tubes containing heparin was centrifuged at 1,500×g for 10 minutes, removing plasma and buffy coat. Erythrocytes were then washed three times with PBS (150 mM NaCl, 10mM Na_2HPO_4, 10mM NaH_2PO_4; adjusted to pH 7.4), suspended in PBS at a hematocrit of 40%, and used on the same day.

(2) Preparation of the antifungal solutions

Amphotericin B was a kind gift of Bristol-Myers-Squibb France (Rueil-Malmaison, France). The drug was first diluted in DMSO at a concentration of approximately 10^{-3} M and then spectrophotometrically adjusted to 10^{-4} M in DMSO (ε416=121 400) and used immediately.

Preparation of salts

Three salt classes from SIGMA prepared with an initial concentration (1 M) were studied:

(1) Chlorides: Potassium chloride (KCl), sodium chloride (NaCl), magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$);

(2) Sulfates: Sodium sulfate (Na_2SO_4), magnesium sulfate ($MgSO_4$), and trisodium sulfate (Na_3SO_4);

(3) Carbonates: Potassium carbonate (K_2CO_3), lithium carbonate ($LiCO_3$), and potassium hydrogenocarbonate ($KHCO_3$).

Evaluation of cytotoxicity of AmB against human red blood cells

After the addition of the antifungal solution to a final concentration of 10^{-5} M and salts with a final concentration of 10 mM, the red blood cells were suspended in buffer PBS, pH 7.4, 10 mM, at a rate of 4000 cells/mL. Erythrocytes, suspension were incubated at 37°C under continuous agitation for 90 minutes.

Aliquots of 500 µL from the reactional solution were carried out in regular intervals, and 2 mL of a frozen solution of washing (NaCl 150 mM, $MgCl_2$ 2mM) was added.

After centrifugation (1,500×g for 5 minutes), the supernatant was recovered, intracellular potassium (K^+), which escaped from the cell, was tested by a flame photometer (JENWAY/PFP7), and hemoglobin was obtained by optical density at 548 nm.

Evaluation of the antifungal activity of AmB

Candida albicans ATCC10231 cultures were carried out in YM liquid medium (pH 6.2 ± 0.2) in the presence and absence of the antifungal solution. The cellular starting concentration is 2×10^6 cells/mL.

The antifungal solution was added to the culture medium at a final concentration of 10^{-6} M at the same time as the inoculum, which corresponds to time zero of the growth. Salts were also added to time zero of the growth at a final concentration of 10^{-2} M.

The cultures were carried out on YM in sterile tubes of 50 mL containing 30 mL of culture medium. Incubation was carried out in an incubator of bend (Bioblock Scientific ROTATEST) with 30°C under agitation continuously with 130 tours/minute. The measurement of

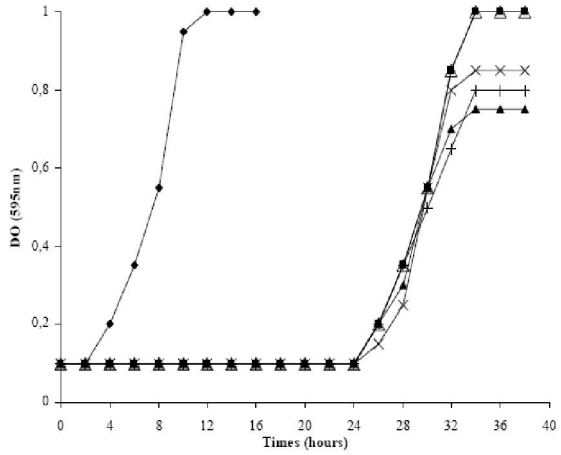

Figure 5 : Influence of 10^{-2}M of chlorates on the activity of AmB (final concentration at 4×10^{-7}M) toward the growth of *Candida albicans*. Inoculums of 2×10^{6} cells/mL. Incubation at pH 6.2 and 30°C in continuous agitation. (-◆- Control, -■- DMSO, -▲-$MgCl_2$, - ×- NaCl, -+-KCl, -△-$CaCl_2$)

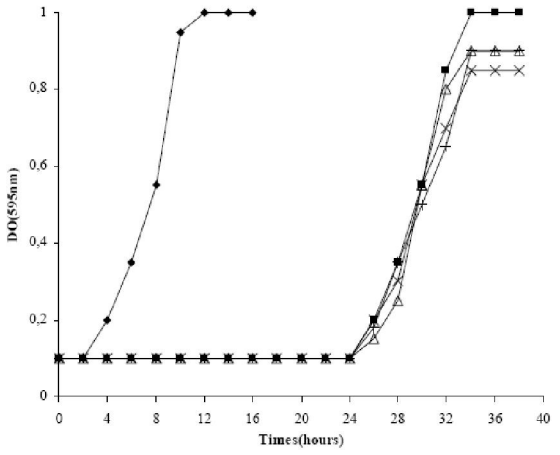

Figure 6 : Influence of 10^{-2}M of sulfates on the activity of AmB (final concentration at 4×10^{-7}M) toward the growth of *Candida albicans*. Inoculums of 2×10^{6} cells/mL. Incubation at pH 6. 2 and 30°C in continuous agitation. (-◆- Control, -■- DMSO, -△-K_2SO_4, - ×- $MgSO_4$, -+-Na_2SO_4)

the growth was performed by cellular numeration on Malassez cells every 90 minutes.

RESULTS

Effect of the addition of salts on the intra-erythrocyte K^+ leakage

Addition of the chlorides (NaCl, KCl, $CaCl_2$, and $MgCl_2$), sulfates (K_2SO_4, $MgSO_4$, and Na_2SO_4), or carbonates ($LiCO_3$, $KHCO_3$, and K_2CO_3) to the erythrocyte suspension in the absence of AmB does not induce hemolysis or any disturbance of intracellular K^+. Moreover, pH of the medium is maintained at 7.4.

In the presence of 10^{-5} M of AmB, the erythrocytes were emptied completely of their K^+ after 5 minutes of incubation. This potassium escape was reduced by 10% after the addition of salts such as NaCl, KCl, and $CaCl_2$ at a rate of 10^{-2} M. On the other hand, the addition of $MgCl_2$ under the same experimental conditions allows a significant reduction of this leakage of K^+; the red blood cells preserve 70% of their potassium contents during 90 minutes of follow-up (Figure 1).

In the presence of carbonates ($LiCO_3$ and $KHCO_3$) at 10^{-2} M in the reactional medium, the escape of potassium, induced by AmB, was reduced by 30% and 20%, respectively (Figure 2).

Figure 3 indicates that $MgSO_4$ and Na_2SO_4, at a rate of 10^{-2} M in the reactional medium, reduce the leakage of K^+ by 10% after 90 minutes of incubation.

This leakage of K^+ is parallel to the loss of hemoglobin. Indeed, this loss is about 70% in the presence of KCl, $CaCl_2$, and Na_2SO_4, 65% with NaCl, 50% in the presence of $MgSO_4$ and Na_2SO_4, 40% in the presence of $LiCO_3$ and $KHCO_3$, and 35% in the presence of $MgCl_2$ (data not shown).

Effect of addition of salts on the antifungal activity of AmB

The addition of $MgSO_4$, NaCl, KCl, K_2SO_4, or $MgCl_2$ at a rate of 10^{-2} M in the medium culture and in the absence of AmB inhibits the growth of *Candida albicans* ATCC10231 by 20% to 60%, compared to a pilot culture without any addition. $CaCl_2$ completely inhibits the yeast growth. This inhibition is maintained for 24 hours; then the cells again take a growth similar to the pilot culture.

It should be noted that, in the absence of AmB, the addition of sulfates and chlorides in the medium culture does not induce any variation of the pH which is maintained at 6.2. On the other hand, the addition of carbonates in the medium culture moves the pH toward 8.4. Because of the medium alkalization, and under these experimental conditions, the growth of yeasts is completely inhibited. This growth inhibition is irreversible as the pH of the medium is maintained at 6.2; the yeasts prolong their latency phase beyond 24 hours (Figure 4).

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With 4×10^{-7} M of AmB, the yeasts are in the latency phase for 24 hours; then they multiply in a way similar to the pilot cells in the absence of an antifungal. This resumption of growth is related to the dormancy of yeasts (Boucherit and al., 2007).

The addition of chlorides in the culture medium with a final concentration of 10^{-2} M increases the effectiveness of this drug. Improvement varies according to salt used. Indeed, it is 20% in the presence of $MgCl_2$, 15% in the presence of KCl, and only 10% in the presence of NaCl (Figure 5). Contrary to chlorides, the addition of sulfates to the drug does not induce any significant improvement in antifungal activity (Figure 6).

DISCUSSION

The work we undertook focused on the in vitro determination of the effect of the addition of some salts on the selective activity of AmB. For tests of cytotoxicity, we used a universal model of animal cells (i.e., the human red blood cells coming from a healthy single donor). While being fixed at membrane sterols, AmB induces a disturbance of the permeability of the cells. The first indicating element of this disturbance is the leakage of intracellular K^+, followed by that of other cellular metabolites and lyses (Bolard and al., 1991). At the red blood cell, lyses results in the release of hemoglobin. The antifungal effectiveness of this drug was evaluated on *Candida albicans* ATCC10231, yeast responsible for 80% of the fungal infections.

According to the results obtained, it seems that the toxicity of AmB toward the animal cells is not modulated by the ionic environment of the antifungal one. Indeed, the toxicity of the antifungal was reduced by 10% only in the presence of sulfates and chlorides; the antifungal effectiveness against yeasts is maintained.

Although the carbonates allowed a significant improvement in the therapeutic index of AmB, the significant shift of pH (from 5.6±0.2 to 8.3±0.2) caused by their addition to the culture medium is a major disadvantage, as the pH is also a significant factor in the modulation of antifungal activity[5].

Only $MgCl_2$, used with a final concentration of 10^{-2}M, protects the human red blood cell from the toxic effect of AmB; the erythrocytes lose only 30% of their K^+. This protection is maintained for final concentrations of AmB going until 2×10^{-5} M, which corresponds to 20 times the inhibiting minimal concentration. Moreover, this same salt increases the effectiveness of the AmB against *C albicans* by 10%.

$MgSO_4$ reduces the toxicity of AmB against the red blood cells by 10%. The difference existing between $MgCl_2$ and $MgSO_4$ could be due to the fact that SO_4^{2-} reduces the solubility of AmB in aqueous medium; this supports the formation of toxic oligomers of AmB for the animal cells[24]. In addition, SO_4^{2-} is less permeable than Cl^-[18].

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