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Original article

Amphotericin B mediates killing in *Cryptococcus neoformans* through the induction of a strong oxidative burst

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Abstract

We studied the effects of Amphotericin B (AmB) on *Cryptococcus neoformans* using different viability methods (CFUs enumeration, XTT assay and propidium iodide permeability). After 1 h of incubation, there were no viable colonies when the cells were exposed to AmB concentrations $\geq$ 1 mg/L. In the same conditions, the cells did not become permeable to propidium iodide, a phenomenon that was not observed until 3 h of incubation. When viability was measured in parallel using XTT assay, a result consistent with the CFUs was obtained, although we also observed a paradoxical effect in which at high AmB concentrations, a higher XTT reduction was measured than at intermediate AmB concentrations. This paradoxical effect was not observed after 3 h of incubation with AmB, and lack of XTT reduction was observed at AmB concentrations higher than 1 mg/L. When stained with dihydrofluorescein, AmB induced a strong intracellular oxidative burst. Consistent with oxidative damage, AmB induced protein carbonylation. Our results indicate that in *C. neoformans*, Amphotericin B causes intracellular damage mediated through the production of free radicals before damage on the cell membrane, measured by propidium iodide uptake.

Keywords: *Cryptococcus neoformans*; Amphotericin B; Oxidative burst; XTT; Propidium iodide

1. Introduction

Amphotericin B (AmB) is the antifungal agent that has been most widely used in clinical practice since its discovery in 1955 [1]. It presents a potent activity against the majority of fungi, including yeast and filamentous moulds (see review in [2]), and only a few species, such as *Aspergillus terreus*, *Scedosporium prolificans* and *Trichosporon asahii*, show intrinsic resistance to this compound [2–4]. Classically, its main action mechanism has been binding to ergosterol, which yields pores in the membrane, ion loss and cell death (see review in [5]). Although in the last years new antifungals have been included in clinical practice, such as azole derivatives and candins, AmB still remains as the first choice for the treatment of different invasive mycoses, such as zygomycosis and cryptococcal meningitis (see reviews in [6,7]). This last disease is caused by the pathogenic fungus *Cryptococcus neoformans*, whose incidence has significantly emerged in the last decades, especially among HIV patients. Although in developed countries its incidence has decreased due to the introduction of the high active antiretroviral treatment (HAART), its early associated mortality has not changed [8,9]. Moreover, in developing countries, it has been estimated that

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this fungus causes more than 600,000 deaths per year [10]. The treatment for cryptococcal meningitis is based on an initial therapy of Amphotericin B followed by a maintenance treatment with fluconazole (see recent guidelines in [11] and review in [12]). Resistance to these compounds has been described among clinical strains [13] and for this reason, investigation of the role of Amphotericin B on *C. neoformans* has particular interest.

Although binding to ergosterol and pore formation is the most accepted mechanism of action for AmB (see review in [14]), several reports suggest that this antifungal has other effects on the cell. In particular, it has been suggested that AmB can be auto-oxidized with the subsequent production of free radicals [1,15,16]. In this work, we have measured the effect that AmB has on the viability of *C. neoformans* using different methods: XTT reduction assay, propidium iodide staining, and CFUs enumeration. In the XTT assay, a tetrazolium salt is added to the cells, and this compound is reduced to formazan by living cells [17,18]. This phenomenon occurs mainly through electron transfer at the mitochondrial level. In the case of propidium iodide staining, this compound is taken by the dead cells as a consequence of a disruption of the membrane integrity, which results in an increased permeability and fluorescence in dead cells once it binds to DNA, which is proportional to the DNA content of the cells [19]. Finally, the long-term effect of an antifungal on the ability of the cells to yield a viable colony (colony forming units, CFUs enumeration) is also widely used, and reflects the final outcome of the interaction between the compound and a cell.

Our results indicate that after AmB addition, the cells become metabolically inactive before they lose their membrane integrity. Moreover, AmB rapidly induces a strong oxidative burst in the cells, which suggests that AmB has other effects on *C. neoformans* different to binding to the cell membrane and killing induction through pore formation.

2. Material and methods

2.1. Yeast strains and growth conditions

C. neoformans H99 strain (serotype A, [20]) was the strain used in most of the experiments. In addition, 24067 (ATCC), B3501 [21] and other *C. neoformans* strains from the Yeast Collection of the Spanish National Mycology Reference Laboratory (CL-2132, CL7041, CL5801) were also used. The yeasts were routinely grown in Sabouraud liquid medium overnight at 30 °C with moderate shaking (150 rpm). Then, the cells were collected by centrifugation, and suspended in PBS. To induce melanization, cells from H99 strain were grown in L-Dopa medium (25 mM L-3,4-dihidroxyphenylalanina, 20 mg/mL thiamine-HCL, 29.4 mM KH_2PO_4, 10 mM $MgSO_47H_2O$, 13 mM Glycine, 15 mM D-Glucose) for 7 days at 30 °C with shaking. To obtain non-melanised control cells, the yeasts were grown in the same conditions without L-DOPA.

2.2. Amphotericin B-induced killing

Yeast cells were grown as described above and suspended in PBS buffer at 10^7 cells/mL (2× stock). Then, Amphotericin B (Sigma–Aldrich Quimica, S. A. Madrid, Spain) was diluted to 2× stock aliquots, and equal volumes of cells and AmB were mixed. The final AmB concentrations ranged from 0.06 to 16 mg/L. The cells were incubated at 37 °C during different time periods (1, 3 or 24 h), and aliquots were plated in agar Sabouraud plates. The plates were incubated at 30 °C during 48 h, and the number of colonies was enumerated. The viability was expressed as percentage of colonies obtained in the samples treated with Amphotericin B compared to the untreated control.

2.3. XTT assay

Cells from H99 strain were grown in liquid Sabouraud at 30 °C for different time periods (24 or 48 h). Then, the cells were extensively washed with PBS, and counted using a haemocytometer. A suspension of 10^7 cells/mL was prepared in PBS, and 100 μL of this suspension were added to each well of 96-well microdilution plates. Then, Amphotericin B aliquots were prepared as 2× stocks, and 100 μL were added to the wells. As a result, 10^6/mL cells were exposed to AmB at different concentrations (from 0.06 to 16 mg/L) in a final volume of 200 μL. One hundred microliters of PBS were added to several wells as control of viable cells. In parallel, an aliquot of the original yeast cells suspension (10^7 cells/mL) were heat-inactivated by incubating the cells at 60 °C during 45 min, and 100 μL of this suspension were also added to different wells (killed cells controls). The microdilution plate was incubated at 37 °C for different time periods (1 and 3 h), and then centrifuged at 3500 g. Then, the supernatant was removed, and XTT reduction by metabolically active cells was measured as described in [22]. Briefly, 100 μL of a solution containing 0.5 mg/mL of 2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Sigma–Aldrich) and 25 μM menadione (Sigma–Aldrich, prepared from a 10 mM stock solution in absolute ethanol) in PBS were added to each well. Since XTT reduction by living cells is not an end-point reaction, the absorbance at 450 nm was monitored over time. The plate was incubated at 30 °C in an iEMS Reader MF spectrophotometer (LabSystems, Thermo Fisher Scientific) and the optical density at 450 nm was monitored every 30 min during 18 h. Viability was calculated from the absorbance values obtained after 4–6 h of incubation. The value of the average optical density of the heat-killed cells was subtracted from the optical density of the rest of the wells. Then, the viability percentage was calculated as the (O.D of the amphotericin treated wells/O.D. of the viable cells) × 100. All the experiments were repeated in different days, obtaining very similar results.

2.4. Propidium iodide staining

The cells were prepared and treated with Amphotericin B as described above (Amphotericin B-induced killing section),

and propidium iodide was added at 10 μg/mL. Then, the fluorescence intensity was measured by flow cytometry using a FACSCalibur cytometer (Becton Dickinson) and the CellQuest software in a Macintosh computer. The data was finally processed using the WinMDI 2.9 software (http://facs.scripps.edu/software.html). Heat-killed and viable cells (non-treated with amphotericin B) were also included. The percentage of dead cells in each sample was defined as the percentage of cells stained with propidium iodide compared to the total number of cells analyzed in that sample. For the analysis, no gate of the FSC/SSC plot was used, so all the cells were considered in our study.

2.5. Detection of free radicals by dihydrofluorescein diacetate (DHF) treatment

C. neoformans cells (5×10^6/mL) were treated with different concentrations of hydrogen peroxide (H_2O_2) or AmB during 1 or 3 h. Then, DHF was added from a 100× stock solution to the cells to a final concentration of 40 μM. Intensity of fluorescein-formed fluorescence was measured in a FACSCalibur cytometer using the CellQuest software, and the data was finally processed using the WinMDI 2.9 software (http://facs.scripps.edu/software.html). For the analysis, no gate of the FSC/SSC plot was used, so all the cells were considered in our study.

2.6. DAPI staining and fluorescence microscopy

To observe the nuclei, the cells were treated with 2 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI). After 2 min incubation at room temperature, the cells were washed with PBS and placed on a slide. Then, fluorescence was observed under a Leica DMI3000B. Pictures were taken with a Leica DFC300 digital camera using the LAS 3.3.1 software. Figure compositions were made using Adobe Photoshop 7.0 software.

2.7. Detection of protein carbonylation

Analysis of protein carbonylation after derivatization of carbonyl groups with dinitrophenylhydrazine (DNPH) was carried out as described in [23]. Blotted membranes were incubated with rabbit anti-DNPH antibodies (Sigma), at 1:2000 dilution. The experiment was repeated twice with different samples collected in different days, obtaining very similar results. Quantification of the intensity of the bands was performed using ImageJ software (National Institute of Health, http://rsb.info.nih.gov/ij). A selection covering a representative area of the wells was selected, and a relative plot of the intensity of the protein of those wells was calculated using the software. This was done for both the intensity of the carbonylated proteins, as well as for the control gel stained with Coomassie. The area of the intensity of each well was calculated, and the relative intensity was estimated as the intensity of the well of the carbonylated protein divided by the intensity of the well of the Coomassie gel. Then, for each time point, the control well (without any treatment) was giving an arbitrary value of 100, and the value for the treated samples was expressed as percentage respected to the value of this control well.

3. Results

3.1. Correlation between CFUs and propidium iodide uptake after AmB treatment

To study the effect of Amphotericin B on *C. neoformans*, we compared the fungicidal activity using three different methods: CFUs enumeration, XTT reduction by living cells and propidium iodide staining. While comparing and standardizing these protocols, we observed discrepancies in the viability calculated. When *C. neoformans* cells were exposed to AmB and aliquots were plated, there was clear decrease in the number of viable cells at concentrations above 0.25–0.5 mg/L and a total fungicidal effect at concentrations higher than 1 mg/L (Fig. 1). We also compared the mortality of *C. neoformans* cells in the presence of AmB by another classical viability method, which is the uptake of propidium iodide by dead cells. This dye allows the determination of the cell cycle state of the cells, being easy to observe the typical G1/G2 peaks (Fig. 2, heat-killed control). When we performed a killing assay with different AmB concentrations and observed the fluorescence of the cells after propidium iodide addition by flow cytometry, we observed big discrepancies with the results obtained by CFUs enumeration. While after 1 h of incubation with high AmB concentrations there was a significant degree of killing using the CFUs methods (more than 95% of death at concentrations above 0.5 mg/L), the cells were not permeable to propidium iodide in the same conditions (Fig. 2), and only after 3 h PI uptake was observed in 50–80% of the cells at AmB concentrations above 4 mg/L (Fig. 2). Interestingly, the fluorescence intensity signal of the cells treated with high AmB concentrations for 3 h was higher than the signal obtained with heat-killed cells. This finding suggests that AmB could have effects on the DNA conformation or structure, so this molecule would able to bind higher

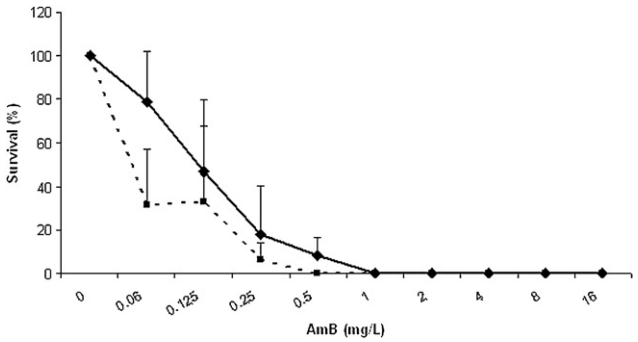

Fig. 1. Killing effect of AmB measured by CFUs enumeration. H99 strain was incubated with different AmB concentrations for 1 (solid line) or 3 h (dotted line). After the incubation, aliquots of the cell suspensions were plated on solid Sabouraud media for 48 h, and the number of CFUs was enumerated after 48 h. The graph represents the average and standard deviation of three different experiments.

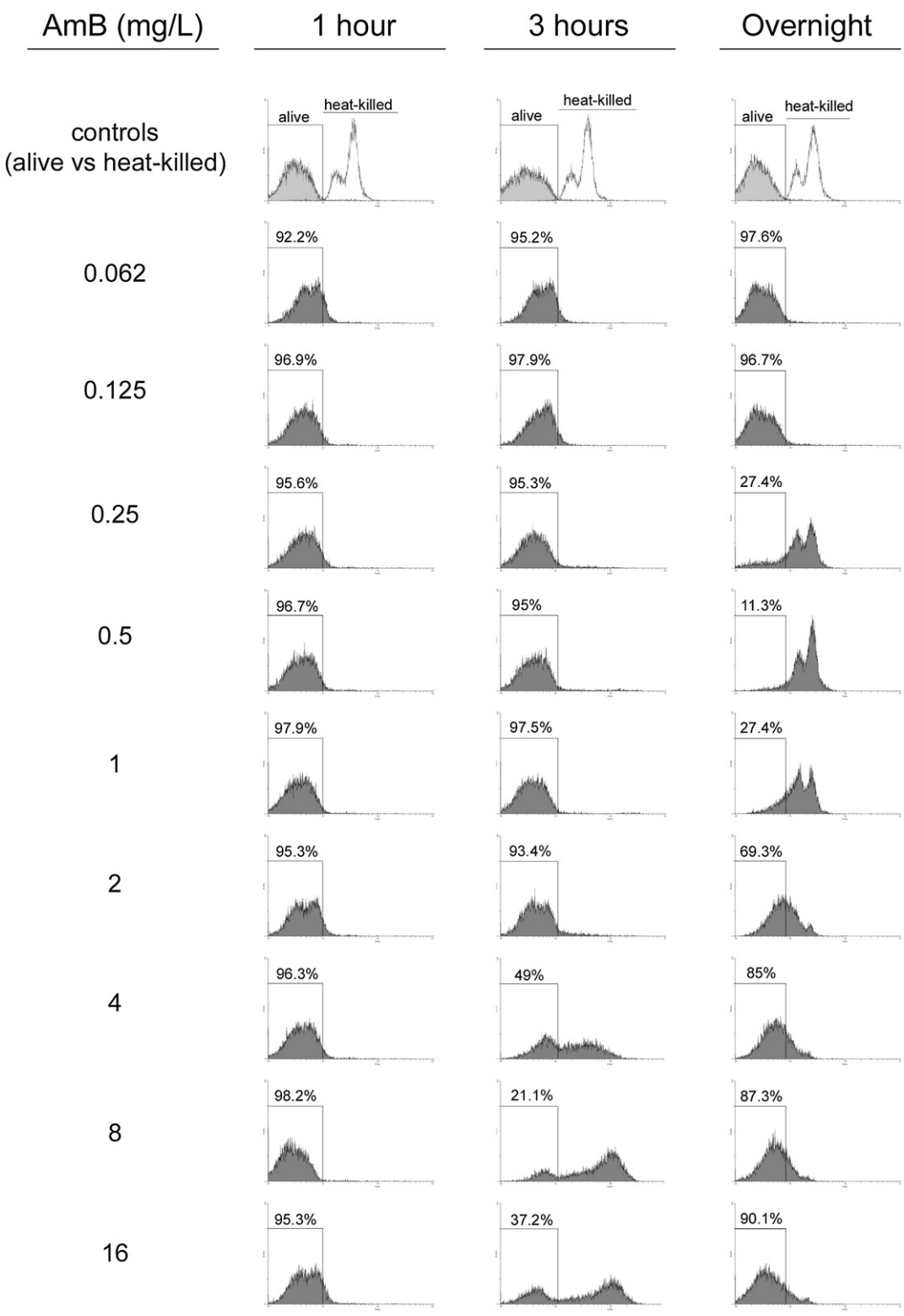

Fig. 2. Propidium iodide staining of Amphotericin B-treated cells. Cells from H99 strain were treated with different AmB concentrations for 1 or 3 h or overnight, and then propidium iodide was added as described in Material and methods. The proportion of PI positive cells was determined by flow cytometry. Control samples of untreated (negative control) or heat-killed cells (positive control) were included. Using these controls, it was possible to establish the areas of PI-negative cells, which is presented in each plot.

amounts of PI on AmB-treated cells than on regular cells. Curiously, when the cells were incubated overnight with AmB, PI staining was observed only at intermediate concentrations, and cells incubated with the higher concentrations became negative for PI staining (Fig. 2). We argued that this lack of signal in those samples was not related to a lack of permeability of the cells, but rather to a lack of nucleus as a consequence of prolonged incubation in the presence of the antifungal. To test this possibility, we stained the cells with another nuclear dye, such as DAPI. When the cells were incubated overnight with 16 mg/L AmB, no clear staining with DAPI was observed. The cells presented a very diffuse fluorescence which rapidly quenched and disappeared, which was different from the signal elicited by control untreated cells, in which clear nuclei were observed. In contrast, when the cells were treated with a low AmB dose (0.12 mg/L), clear nuclei were observed (Supplemental Fig. 1). This data indicates that prolonged incubations with AmB yielded in the destruction of cellular compartments, such as the nuclei, and release of material to the extracellular space.

3.2. *Effect of AmB on* C. neoformans *measured by XTT reduction assay*

We also studied the effect of AmB on *C. neoformans* using the assay based on the reduction of XTT by living cells. In agreement with CFUs, a clear decrease in the reduction of XTT was observed at concentrations higher than 0.5 mg/L. However, we also found a paradoxical effect, in which there was not a linear correlation between the XTT reduction and AmB-induced killing observed by CFUs determination. When AmB concentration was gradually increased and reached concentrations between 2 and 16 mg/L, there was a partial increase in the amount of reduced XTT. We observed a high variation between different experiments with respect to the appearance of this paradoxical effect. So we argued that the initial metabolic state of the cells could have a profound influence on the pattern of XTT reduction. For this reason, we compared the effect of different concentration of AmB on the XTT reduction on cells obtained at different growth phases. To obtain these different growth phases, parallel liquid cultures were inoculated with different number of cells, so the next day they were collected at different growth phases. As predicted, we observed that this paradoxical effect correlated with the initial inoculum of the culture. The paradoxical effect was not observed when the culture was inoculated with 10^3 cells/mL. However, when the concentration of the initial inoculum increased, the paradoxical effect was prominent (Fig. 3A), being maximum when the culture was initially inoculated with 10^6 cells/mL. In another experiment, we inoculated a liquid culture with an initial inoculum of 10^3 cells/mL, and collected samples after 24 and 48 h, which correspond to logarithmic and stationary phase, respectively. In agreement with our previous findings, we observed that the paradoxical effect was absent at 24 h of growth, but obvious after 48 h (Fig. 3B). These results confirm that the paradoxical profile of XTT reduction was dependent on the growth phase of the culture. In any case, we did not obtain viable colonies when the cells were treated with AmB concentrations above 1 mg/L, indicating another discrepancy between the viability measurement methods.

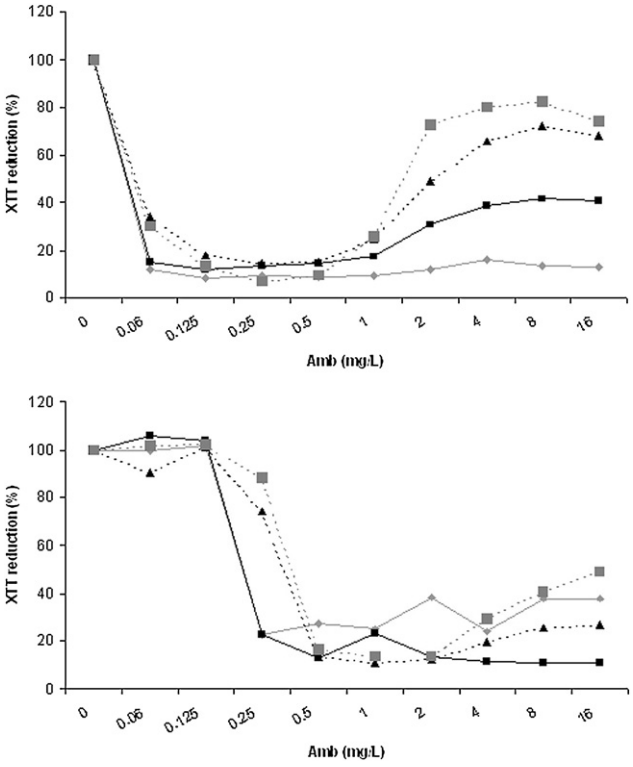

Fig. 3. XTT reduction of *C. neoformans* cells after treatment with different AmB concentrations. (A) *C. neoformans* cells were inoculated at initial concentrations of 10^3 (solid gray line, rhombus), 10^4 (solid black line, squares), 10^5 (black dotted line, triangles) and 10^6 (gray dotted line, squares) cells/mL. Then, the cultures were incubated at 30 °C overnight. Then, the four cultures were collected, washed with PBS, and suspended at 10^7/mL. One hundred microliters of this suspension were placed in 96-well plates, and then AmB was added at different final concentrations, ranging 0.032–16 mg/L. Control wells without AmB were carried out in parallel. The plate was incubated at 30 °C for 1 h, then centrifuged and the medium removed. XTT reduction was measured as described in Material and methods. The percentage of XTT reduction in each well was calculated using the control without amphotericin as a reference value of 100% of XTT reduction. The experiment was performed in duplicate, obtaining from them almost identical results. The plot shows the average of the duplicates. In addition, the experiment was also repeated in different days obtaining very similar and consistent results. (B) A liquid culture was inoculated with cells from H99 strain at an initial cell density of 10^3 cells/mL. The culture was incubated for 24 (dotted lines) or 48 h (solid lines) at 30 °C. Then, the cells were exposed to different AmB concentrations for 1 h and XTT reduction was measured as described in Material and methods and in the section (A) of this figure legend. The experiment was performed in duplicate, both of them shown in the plot.

3.3. *Detection of intracellular free radicals using DHF*

We argued that the paradoxical effect observed in the reduction of XTT by *C. neoformans* cells after addition of high AmB concentrations might be related to a strong induction of metabolic activity in the cells. For this reason, we investigated if AmB produced an oxidative burst. For this purpose, we

treated the cells with dihydrofluorescein diacetate (DHF), which is cleaved into fluorescein by free radicals, producing a fluorescent signal. As shown in Fig. 4, when the cells were treated with AmB and then with DHF, there was a significant increase in the fluorescence of the cells, which was similar to the fluorescence obtained after treatment with H_2O_2. Moreover, the production of free radicals was beginning to be observed at concentrations that are similar to the MIC value (0.25–0.5 mg/L), which suggests that the induction of free radicals participate in the inhibitory effect measured after AmB addition in the standard antifungal tests.

The production of free radicals was not observed when AmB and DHF were added to heat-killed cells, indicating that the effect was dependant on the metabolic activity of the cells and not to a direct effect of AmB on DHF (results not shown). In addition, no fluorescence was detected when the cells were treated with AmB or H_2O_2 without addition of DHF, confirming that fluorescence was due to an induction of free radicals production by AmB and not to auto-fluorescence. We observed these cells under the fluorescence microscope, and confirmed that AmB induced a strong cleavage of DHF in the cells (result not shown). In addition, we wanted to discard that a prolonged incubation in PBS could result in cellular changes that affected our results. For that purpose, we measured the oxidative burst produced in cells preincubated in PBS for 3 h. When we placed the cells in PBS for this time, and then added AmB, the production of free radicals was very similar to those conditions in which the cells were not preadapted (data not shown), suggesting that the incubation medium did not have a significant influence at the cellular level that could influence the production of free radicals. We also studied if AmB induced free radicals accumulation in media containing serum, to mimic the in vivo situation. When we added 10% of serum to the buffer, AmB induced the production of free radicals in a similar way than in the regular buffer, suggesting that the oxidative burst might be occurring also in vivo. To confirm that the phenomenon was not strain-specific, we studied the production of free radicals in 6 different strains after addition of AmB. All the strains produced free radicals when exposed to AmB (result not shown).

As stated above, we observed that XTT reduction had a paradoxical effect when the cells were treated with high AmB concentrations. We decided to investigate if this effect was related to a different effect of the production of free radicals. We inoculated different cultures with 10^3 and 10^6/mL cells, and after 24 h, examined their susceptibility to AmB. These cultures exhibited different susceptibility to AmB, being the culture inoculated with 10^6/mL slightly more resistant measured by CFUs determination (result not shown). We studied the effect of AmB different concentrations on the production of free radicals and on the uptake of PI. We chose a low (0.125 mg/L), intermediate (0.5 mg/L) and high (8 mg/L) concentration, based on the different susceptibility of the yeast shown at these concentrations. As shown in Fig. 5, there was also a paradoxical effect on the production of free radicals in these conditions. Low AmB concentrations induced a strong production of free radicals after 3 h of incubation in the culture inoculated with 10^3 cells/mL. In contrast, when we exposed cultures to intermediate AmB concentrations, we observed a paradoxical effect after 1 h, since in this case production of free radicals was prominent in the culture inoculated with a high dose (10^6/mL), but not with a low dose (10^3/mL). This effect did not correlate with viability, measured by PI uptake (Fig. 5) or CFUs enumeration, but with the paradoxical effect observed with the XTT reduction assay. In any case, when a high AmB concentration was used (8 mg/L), a strong oxidative burst was observed.

3.4. Effect of AmB on protein carbonylation and cellular structure

Free radicals mediate damage in the cells through multiple mechanism, being one of them protein carbonylation [24]. We tested if AmB induced this protein modification by western blot. As shown in Fig. 6A and B, AmB induced protein carbonylation. When we performed a quantification of the amount of carbonylated proteins in each condition (Fig. 6C), we observed that after 1 h of incubation there was a slight increase in the amount of carbonylated proteins (around 1.5 fold increase), which was more noticeable after 3 h (over 2-fold increase). AmB-induced protein carbonylation was always lower compared to the samples treated with 5 m H_2O_2, which was included as a positive control of the assay. After a longer incubation time (6 h), protein carbonylation decreased compared to the shorter times, most probably due to the high degree of killing and cellular damage shown by the cells at this time point. In addition to protein carbonylation, we observed that AmB addition resulted in the condensation of intracellular structures, an effect that has been associated with oxidative damage in the cells (Supplemental Fig. 2).

3.5. Effect of AmB in melanised cells

Accumulation of melanin at the cell wall level reduces the susceptibility of *C. neoformans* to AmB [31]. Since melanin has antioxidant properties, we decided to study if there was a correlation between the susceptibility of melanised cells to AmB and the production of free radicals. To induce melanin accumulation, we grew the cells in minimal medium supplemented with L-DOPA, and compared the production of free radicals and propidium iodide uptake with cells grown in the same conditions without L-DOPA. We consistently found that after AmB treatment, the proportion of cells in which AmB induced oxidative burst was lower in the melanised samples (around 7–9% difference), suggesting that the protective role of melanin might be related in part to its antioxidant role.

4. Discussion

Despite AmB has been successfully used in clinical practice during decades for the treatment of fungal infections, its action mechanism has not been completely elucidated. It has been shown that AmB binds to ergosterol at the cell membrane level, where it induces pore formation and loss of cellular integrity. However, there are numerous findings suggesting

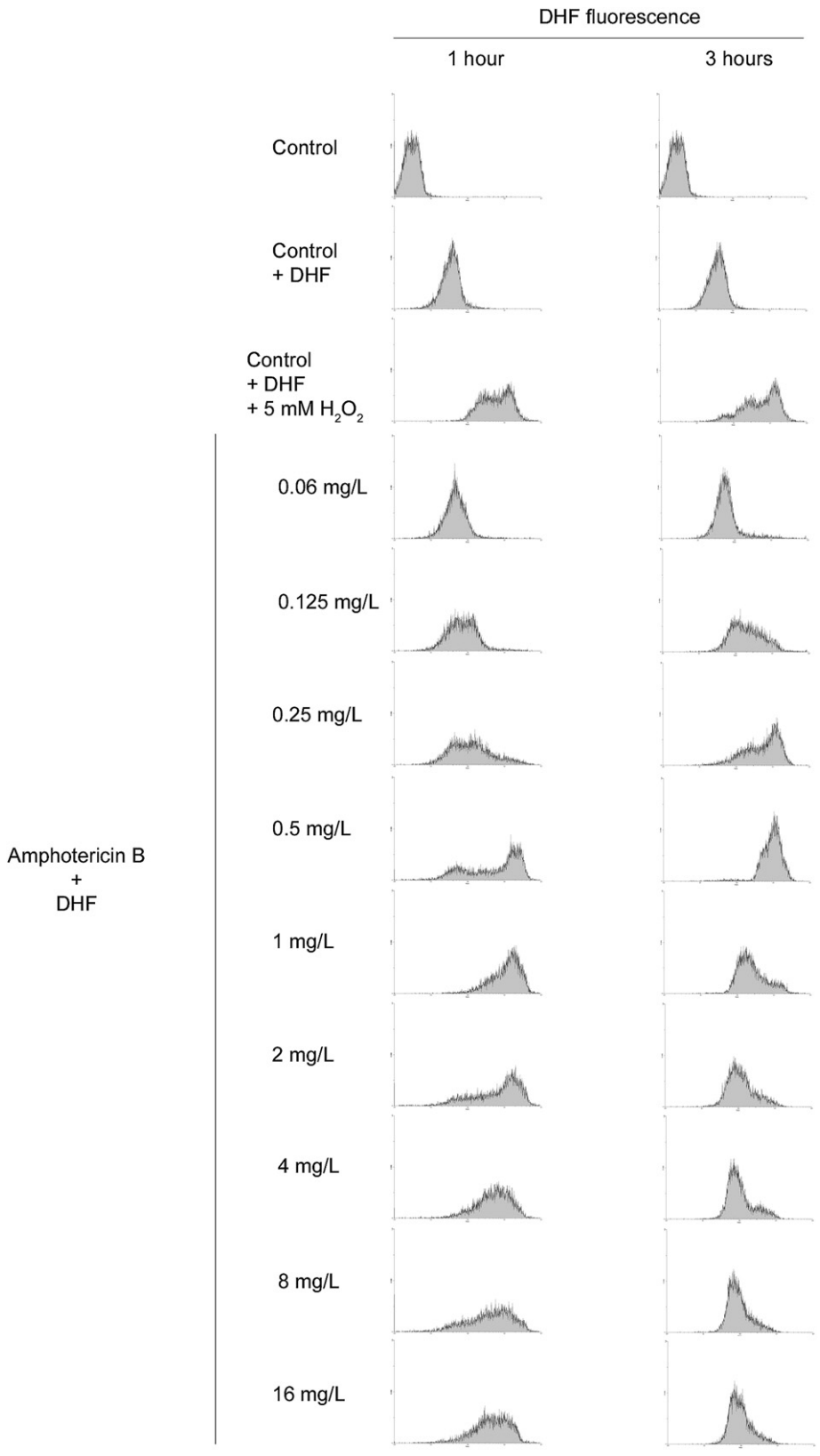

Fig. 4. Effect of AmB on the production of free radicals. *C. neoformans* cells were grown overnight and exposed to different AmB concentrations for 1 and 3 h as described in Material and methods. As controls, the cells were exposed to 5 mM H_2O_2. Then, dihydrofluorescein diacetate (DHF) was added to the cells to measure the production of free radicals, and after 30 min incubation, the fluorescence intensity was measured by flow cytometry (FL1 channel). Control cells without DHF were carried out as auto-fluorescence controls. The plots show the histograms of the fluorescence intensity in each condition.

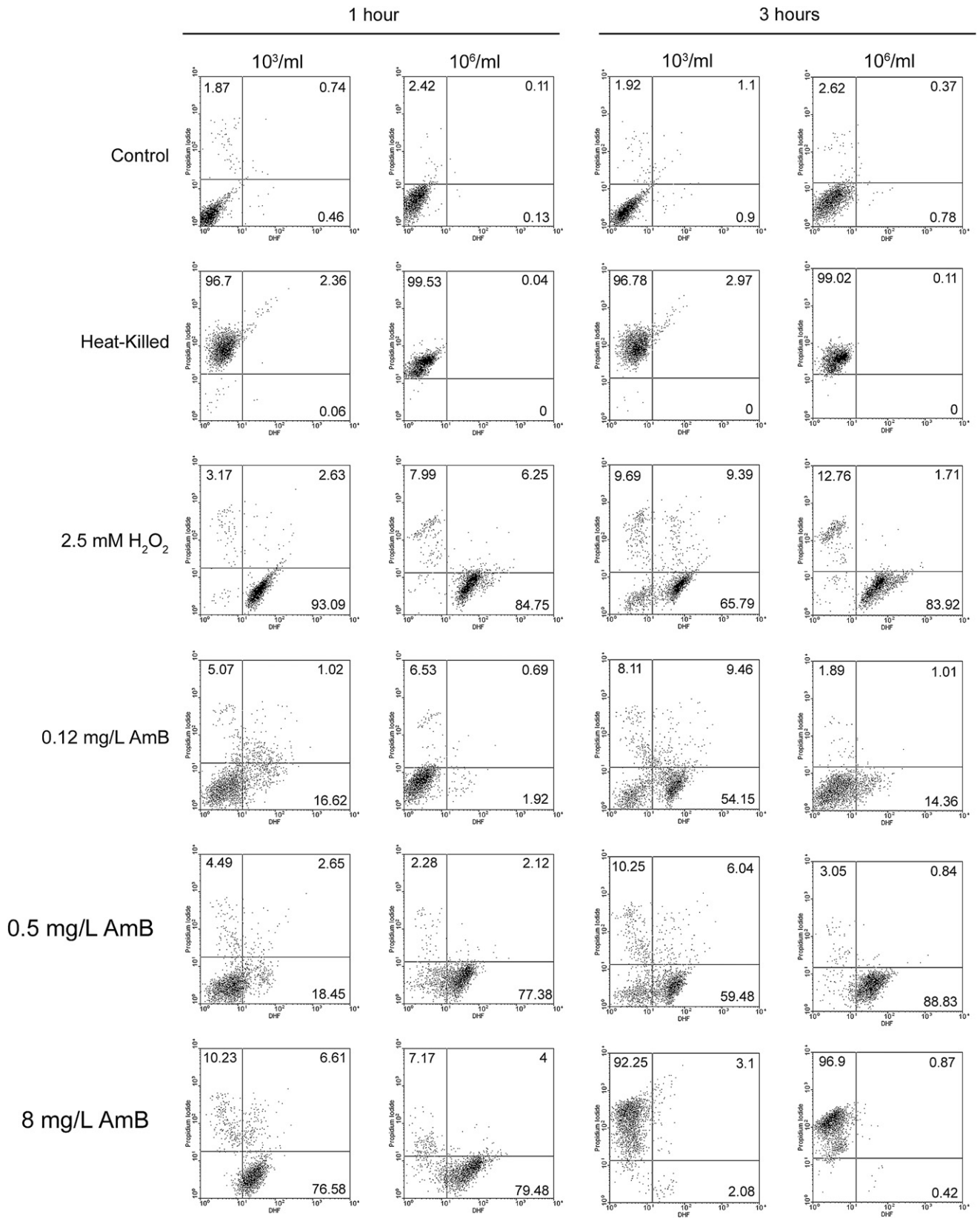

Fig. 5. Free radical production and propidium iodide uptake of cells inoculated at different initial concentrations and treated with different AmB concentrations. Liquid cultures were inoculated at initial cell densities of 10^3 and 10^6 cells/mL. Then, the cells were exposed to different AmB concentrations for 1 and 3 h, and DHF and propidium iodide were added as described in the Material and methods. The fluorescence intensity produced by the DHF cleavage and PI uptake was recorded by flow cytometry. Heat-killed cells, or treated with 2.5 mM H_2O_2, or not exposed to any treatment were carried out in parallel as controls. These controls were also used to determine the quadrants of PI and/or DHF positive cells. The graphs show the dot plots of the fluorescence intensities (*x*-axis, DHF intensity, FL1 channel; *y*-axis, PI intensity, FL3 channel). The numbers indicate the percentage of cells present in each quadrant.

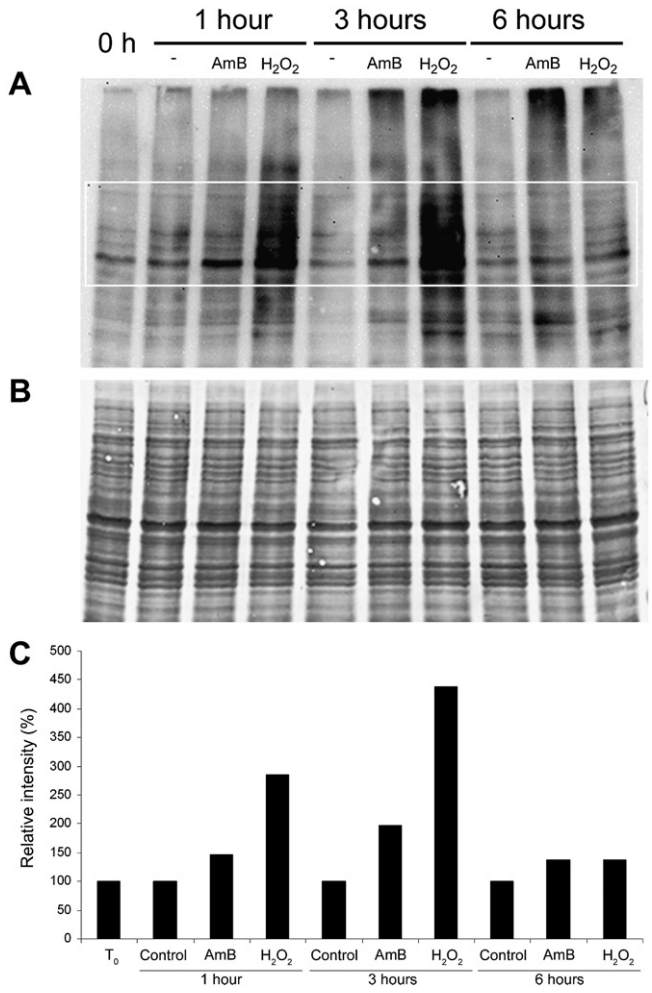

Fig. 6. Detection of protein carbonylation in the presence of AmB. Yeast cells from H99 strain were treated for 1, 3 and 6 h with AmB (1 mg/L) or H_2O_2 (5 mM). Parallel-untreated controls were carried out at each time point. The cells were washed with cold water, and protein extract and western blot were performed as described in Material and methods. (A) Western blot of carbonylated proteins. The region highlighted with a square was the region used to quantify the intensity of the bands shown in panel C. (B) Coomasie staining of the gel (loading control). (C) Quantification of the intensities of the carbonylated proteins normalized by the intensity of the Coomassie gel.

that AmB has other effects on the cell. Early studies indicated that there was a dissociation between the AmB-induced cell death and ion loss, suggesting that pore formation was not the only mechanism involved in the damage produced by AmB in the cells [25]. Recent findings indicate that the ability to form ion channels is not required for the antifungal activity [26]. In *Candida albicans*, it has been shown that AmB induces an apoptosis-like phenotype, a phenomenon mediated through the production of reactive oxygen species (ROS) [27,28]. In agreement with this idea, it has been found that the intrinsic resistance to this antifungal in *A. terreus* is associated with high catalase activity [29], which is consistent with the idea that AmB mediates fungal killing through the induction of an oxidative burst in the cells. In addition, ascorbic acid, which can act both as an anti- or pro-oxidant molecule, enhance the antifungal effects of AmB without increasing the membrane permeability [30].

In this work, we have used several methods that measure different cellular parameters related to viability, and surprisingly, we found significant discrepancies between them. Previous works also indicated that CFU enumeration was not correlated with other viability measurements and that in certain conditions, cells treated with intermediate AmB concentrations could be resuscitated [31], which suggests that AmB has pleiotropic effects on the cells. Surprisingly, we found that the cells became metabolically inactive before losing their membrane integrity, suggesting that the primary effect of AmB on *C. neoformans* does not occur at the membrane level. Moreover, we observed that detection of XTT is not always associated with cell viability, as happened when the cells were treated with high AmB doses for short time periods. In these conditions, high AmB concentrations produced a higher XTT reduction compared to intermediate AmB concentrations, a phenomenon that we consider as paradoxical effect. This effect has been previously shown in the literature using different experimental conditions [31]. The reason for this effect is not clear. It is known that AmB can undergo auto-oxidation, and that this process results in the antifungal inactivation [15]. However, we do not believe that the paradoxical effect is produced by this inactivation, since it is not correlated with an increase in the ability of the cells to replicate and yield viable colonies on solid medium. Since XTT viability method is dependent on the reduction of this compound, and in turn, on the balance of electron transfer in the cells, we argued that this compound could transiently detect significant increases of the ROS production that would lead to cell death. We confirmed these results using dihydrofluorescein, a well-known detector for free radicals. The paradoxical effect of XTT was only observed when the cells reached a certain O.D., a finding which might be related to differences in metabolic activity of the cell. In agreement with this notion, it has been described that the activity of some antifungal compounds depends on the growth phase of the fungus [32].

There are several reports that have shown that AmB induces oxidative burst in the cell [27,28], but the role of this effect on the killing effect has not been determined. Although the oxidative effect of AmB had been previously described, we believe that our work describes important novelties that provide new insights on the AmB action mechanism. The use of multiple AmB concentrations, the study of the effects during time and the use of different viability methods has allowed us to provide the following findings: (1) the effects of AmB on the cell, oxidative burst and propidium iodide uptake, occur at different time points in the same samples. First, AmB induces a strong oxidative burst, and permeability to PI occurs later with time. This suggests that damage on the membrane is a late effect of AmB and that the initial action of AmB occurs intracellularly by inducing strong oxidative burst. Previous articles have shown that AmB had different effects on the cell, so that at intermediate concentrations it produced killing mediated through apoptosis (defined by the intracellular production of free radicals) and at high concentrations through necrosis (defined by the high permeability to propidium

iodide). This data, measured after 200 min [27], is in full agreement with our data in *C. neoformans*. However, in our work, the use of different time points has allowed us to obtain new insights about the effects of AmB. In particular, we have observed that at high concentrations, AmB also induces a strong oxidative burst at shorter time points (1 h). This data indicates that in *C. neoformans*, killing through apoptosis or necrosis are not independent phenomena, but they are observed at different time points. Based on our results, we propose that AmB has different effects on the cell which are measured at different time points. First, AmB would induce an initial and strong oxidative burst on the cells, through an unknown mechanism, and then, it would induce damage on the cell membrane. This later damage would be a consequence of the pore formation, but also by the damage produced by the oxidation of the lipids of the membrane induced by the presence of a high concentration of free radicals. The increase in the permeability of the membrane would occur when the cells are damaged and metabolically inactive by the production of free radicals, so in these conditions, the oxidative burst would not be observed anymore due to the lack of metabolic activity of the cell. For this reason, we prefer to avoid the terms apoptosis or necrosis for the action of AmB on *C. neoformans*, since they are observed in the same cells, but only at different time points, and we prefer to denominate the induction of free radicals as early damage produced by AmB, and the effects on the cell membrane and increased permeability as late damage. (2) AmB induces a paradoxical effect on the reduction of XTT which depends on the initial state of the cells. In this sense, we have observed that the induction of oxidative burst on the cells requires metabolically active cells. This suggests that AmB exerts action by inducing a metabolic pathway involved in electron transfer in the cell. In this sense, free radicals are natural products of multiple enzymatic reactions in the cell, such as the electron chain in the mitochondria. So differences in metabolic state of the cells, such as changes in mitochondrial activity or other enzymes involved in the electron balance in the cell could affect the effect of AmB, and in consequence, determine the appearance of this paradoxical effect. In addition, a striking finding is that AmB-treated cells elicited a stronger fluorescent signal after PI uptake, a finding that may be consistent with a higher DNA degradation by the free radicals productions and in turn, higher PI binding to the DNA. In this sense, previous findings demonstrate that AmB induces DNA condensation [27]. There are several possibilities that explain the effect of AmB on ROS production and on death. It is possible that after AmB addition, the cells detect the binding of the antifungal to the membrane and triggers a signal that produces an apoptotic-like phenotype, with mitochondrial damage and consequent ROS production, which yields later increased permeability and cell death. In this model, both ROS production and increased permeability would be the consequence of the same action mechanism. However, it is also possible that these two effects, ROS production and membrane permeability, are independent phenomena, and that AmB has in fact different targets in the cells. In this context, AmB has been shown to be an oxidizing molecule by itself [15,33,34], and this effect has been related to its toxicity [35]. This could explain the production of an oxidative burst without initially affecting cellular permeability. In this second model, AmB would not have a unique target on the cell. However, even in this second model, the production of free radicals would produce damage on the membrane by lipid peroxidation, so both phenomena, oxidative burst and increased permeability, would finally also be related. In this sense, we have shown that AmB induces protein carbonylation, a modification induced by free radicals. However, this modification was more obvious in the presence of hydrogen peroxide, which suggests that AmB does not only exert its action through the induction of oxidative stress, but also supports the idea that AmB has in fact multiple effects on the cell, some of them mediated through damage on the membrane and some of them through oxidative burst induction. In this context, it has been reported in *C. neoformans* that killing in the presence of other factors, such as some bacteria and stress factors [27,36], is also mediated through the production of free radicals, which suggests that this killing mechanism could be widely distributed.

We believe that our results may have profound consequences in understanding the low clinical resistance observed to this antifungal. According to our findings, AmB elicits its effects on *C. neoformans* through different killing mechanisms. The fact that AmB has multiple effects on the cell indicates that the acquisition of resistance to this antifungal is a complex process that requires the accumulation of several mutations on the genome. We believe that these findings might be also important to understand the intrinsic resistance to AmB of some emergent fungal pathogens, such as *S. prolificans* or some Mucorales species, whose clinical management is complicated due to their particular antifungal susceptibility profile.

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Appendix. Supplementary material

Supplementary material can be found, in the online version, at doi:10.1016/j.micinf.2011.01.015.

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