New Insights Regarding Yeast Survival following Exposure to Liposomal Amphotericin B

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***In vitro* resistance to amphotericin B is an extremely rare event among pathogenic yeasts. However, *in vivo* response is sometimes reduced, resulting in an unfavorable outcome. Such adverse outcomes might be related to subfungicidal plasma concentrations. We aimed to clarify the mechanisms of liposomal amphotericin B (AMB-L; AmBisome)-induced lesions and the mechanisms responsible for yeast cell recovery following exposure at plasma concentrations. The physiological statuses developing following exposure to AMB-L at simulated plasma concentrations (20 to 0.1 mg/liter) and at a constant concentration (3 mg/liter) were assessed in a 24-h time course assay. Time-kill experiments also were carried out under the same AMB-L treatment conditions. Our results suggest that yeast cells develop compensatory responses related to membrane polarization, metabolic activity, and reactive oxygen species (ROS) production after exposure to high plasma concentrations (20 to 5 mg/liter) during the first 6 h; in the remaining 18 h, when exposed to lower concentrations, cells reveal almost full recovery with no evidence of fungicidal activity. In contrast, whenever cells are exposed to a constant concentration above the MIC, despite initially exhibiting compensatory stress responses, soon afterwards they exhibit membrane depolarization, a decrease of metabolic activity, increasing ROS production, and lastly, programmed cell death and necrosis, resulting in succumbing to AMB-L fungicidal effects. This study may represent a step forward in the support of AMB-L use for clinical treatment of invasive fungal infections, since it demonstrates the importance of maintaining levels of AMB-L above the MIC in plasma and tissues to ensure it produces its fungicidal effects.**

The understanding of how fungal organisms respond to antimicrobial therapy is a relevant question both in terms of evolutionary biology and for treatment of invasive fungal infections. During recent decades, fungi have emerged as major human pathogens; *Candida albicans* represents the fourth most common agent of all hospital-acquired infections (1).

Despite over 50 years of use as monotherapy, amphotericin B (AMB) still represents an important therapeutic alternative for the treatment of systemic fungal infections, particularly when infection persists despite treatment with alternative drugs (2). Amphotericin B belongs to the polyene drug class and exhibits a broad-spectrum fungicidal activity. For decades, the prevailing mechanism of action has been that AMB primarily binds to ergosterol, inserts into the cytoplasmic membrane, and forms pore-like structures; the result is osmotic instability, loss of membrane integrity, metabolic disruption, and ultimately cell death (3, 4). Recently, Anderson et al. proposed a new mechanism of action of AMB. Accordingly, amphotericin exists primarily in extramembranous aggregates that kill yeast cells by extracting ergosterol from the plasma membrane. Consequently, membrane ergosterol depletion will interfere not only with cell membrane integrity but also with other cellular processes which highly depend on membrane ergosterol (5).

The development of genetic resistance to AMB among *Candida* species remains extremely rare, in contrast to what is observed with other drugs in the triazole or echinocandin classes (6, 7). However, in spite of the observed high *in vitro* susceptibility (0.125 to 1 mg/liter), the *in vivo* response to AMB is somewhat reduced in about 40% of treated patients (8–10). The exact reasons for this

lower-than-expected response still remain unclear. Some authors do not associate response failure with target modification, as has been observed with other antifungal drugs (11), but with inappropriate concentrations of AMB at the infection site (12). In accordance with this hypothesis, cells exposed to amphotericin B may exhibit different physiological conditions which are related to drug concentration and exposure time (12). In addition, studies of yeast apoptosis have revealed the occurrence of a programmed cell death or a cellular necrotic response depending on the AMB concentration (13, 14).

Considering the clinical relevance of liposomal amphotericin B (AMB-L; AmBisome) and based on plasma levels described previously (15), this study aims to explore yeast survival mechanisms in response to AMB-L in order to identify different physiological conditions following exposure to decreasing concentrations in a time course assay. In addition, we intend to determine whether

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TABLE 1 Yeast strains used in this study^a

ID	Species	Source	MIC (mg/liter)	% of depolarized cells		
				3 h	6 h	24 h
ATCC 90028	<i>Candida albicans</i>	American Type Culture Collection	0.5	61.21	77.42	26.66
596	<i>Candida albicans</i>	Clinical isolate	0.5	33.62	40.16	21.72
590	<i>Candida glabrata</i>	Clinical isolate	1	28.19	37.51	20.04
597	<i>Candida parapsilosis</i>	Clinical isolate	0.5	34.62	45.98	9.57
120	<i>Candida krusei</i>	Clinical isolate	8	31.08	43.29	19.81
514	<i>Candida tropicalis</i>	Clinical isolate	1	48.65	77.92	21.54
479	<i>Candida dubliniensis</i>	Clinical isolate	0.06	21.64	36.73	25.57
520	<i>Candida lusitanae</i>	Clinical isolate	0.25	30.18	33.21	22.94
D1	<i>Cryptococcus neoformans</i>	Clinical isolate	0.5	28.14	35.79	19.31
BY4741	<i>Saccharomyces cerevisiae</i>	American Type Culture Collection ^b	0.5	20.13	45.83	19.67
PYCC 6480	<i>Debaryomyces hansenii</i>	Portuguese Yeast Culture Collection ^b	1	43.78	89.90	30.47
PYCC 4166	<i>Kluyveromyces lactis</i>	Portuguese Yeast Culture Collection ^b	0.06	27.89	38.89	5.63
CBS 732	<i>Zygosaccharomyces rouxii</i>	CBS-KNAW Fungal Biodiversity Center ^b	0.06	14.85	34.71	12.38

^a Liposomal amphotericin B susceptibility test results and membrane potential of yeast cells treated with AMB-L plasma concentrations after 3, 6, and 24 h, expressed as the percentage of depolarized cells.

^b Kindly provided by Catarina Prista, Instituto Superior de Agronomia, Lisbon, Portugal.

cell survival following exposure to AMB-L is a common phenomenon among different yeast species, some of which are relevant clinical pathogens.

Our results provide novel insights regarding mechanisms by which yeast cells can escape AMB-L fungicidal action depending upon the time of exposure and the concentration of the drug.

MATERIALS AND METHODS

Yeast strains and growth conditions. A broad range of pathogenic and nonpathogenic yeasts were grown in yeast extract-peptone-dextrose (YPD) liquid medium (1% yeast extract, 2% peptone, 2% dextrose; Formedium, Norfolk, United Kingdom) at 35°C with shaking (150 rpm) until the exponential growth phase (details are presented in Table 1) (16). All of the strains were subjected to antifungal susceptibility testing, cell viability assays, and membrane potential evaluation. For membrane integrity, metabolic activity, reactive oxygen species (ROS) production, and apoptotic assays, only *Saccharomyces cerevisiae* BY4741 was used as a model organism. Prior to experiments, yeasts were subcultured twice in YPD agar to ensure the purity of cultures.

Liposomal amphotericin B susceptibility. The MIC of AMB-L (provided by Gilead Sciences, Inc., San Dimas, California) was determined according to the M27-A3 protocol and M27-S3 supplement of the Clinical and Laboratory Standards Institute (CLSI) (17, 18). MIC values were determined after 24 and 48 h of incubation with AMB-L; the MIC was the lowest concentration that prevented any discernible growth. Due to a lack of established clinical breakpoints for AMB-L, the *Candida* isolates were considered wild type (wt) whenever the MIC was ≤ 2 mg/liter and non-wild type (nwt) when the MIC was > 2 mg/liter according to the epidemiological cutoff values (ECVs) proposed by Pfaller and Diekema (19). For non-*Candida* genera, only the MIC value is displayed, since amphotericin B ECVs and clinical breakpoints remain undefined (19). The *C. albicans* ATCC 90028 reference type strain was used as recommended by the CLSI protocol.

Evaluation of cell viability. Yeast suspensions (10^6 yeast cells/ml) were exposed to AMB-L in accordance with the plasma levels established by Walsh et al. (15). Briefly, cell suspensions were treated with 10 mg/liter AMB-L for 30 min; afterwards, cells were harvested by centrifugation at $1,610 \times g$ for 10 min at room temperature (Universal 320R; Hettich). The same cells then were resuspended in fresh culture medium and exposed sequentially to serial concentrations of AMB-L: 20 mg/liter (for 30 min), 10 mg/liter (for 2 h), 5 mg/liter (for 3 h), 1 mg/liter (for 6 h), and 0.1 mg/liter (until 24 h) (detailed in Fig. 1A). Another cell suspension was treated with 3 mg/liter AMB-L for 24 h. During that period at 1, 3, 6, and

12 h of treatment, those cells were harvested (Universal 320R; Hettich) and resuspended in fresh medium containing 3 mg/liter AMB-L, similar to the protocol developed for the cells exposed to various dosages of AMB-L (plasma conditions). Following 1, 3, 6, and 24 h from the start of incubation, the number of viable cells was determined by plating on YPD agar and incubating at 35°C for 24 h; the number of CFU was determined and compared to that of the plate control (not exposed to AMB-L). Before being plated, cells were washed and resuspended in fresh medium in order to prevent antifungal carryover.

Functional characterization of liposomal amphotericin B-induced action. The physiological status of yeast cells which developed following AMB-L exposure to decreasing concentrations or a constant concentration of AMB-L in a time course assay were assessed by flow cytometry. A cell suspension corresponding to 10^6 yeast cells/ml was used in all assays described below. Yeast cells were incubated with various concentrations of AMB-L, in accordance with the scheme previously proposed (Fig. 1A). At 1, 3, 6, and 24 h, aliquots were collected and tested; all cytometric evaluations were performed in a standard FACSCalibur cytometer (BD Biosciences, Sydney, Australia) equipped with 3 photomultipliers (PMTs), standard filters, and a 15-mW, 488-nm argon laser using CellQuest Pro software (version 4.0.2). All trials were performed in triplicate.

Assessment of membrane integrity. Cell membrane integrity was assessed with propidium iodide (PI; Sigma-Aldrich, Munich, Germany) staining. After antifungal treatment, yeast cells were stained with 1 mg/liter of PI for 30 min at 35°C at 150 rpm in the dark (20). The fluorescence intensity (FI) was measured at FL3 (630 nm). The amount of injured cells in each sample was defined as the percentage of PI-positive cells.

Assessment of membrane potential. The effect of AMB-L exposure on cell membrane potential was evaluated using bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3); Sigma-Aldrich, Munich, Germany] as described by Teixeira-Santos et al. (21). After AMB-L treatment, cells were incubated for 15 min in the dark at 35°C at 150 rpm with 0.5 mg/liter of DiBAC₄(3). The FI was registered at FL1 (530 nm).

Assessment of metabolic activity. Metabolic changes induced by AMB-L in *S. cerevisiae* were evaluated using 5-carboxyfluorescein diacetate (5-CFDA; Sigma-Aldrich, Munich, Germany) at a 10 μ M final concentration. Antifungal-treated cell suspensions were stained with 5-CFDA and incubated for 45 min at 35°C at 150 rpm in the dark (12). The mean intensity of fluorescence (MIF) was registered at FL1 (530 nm).

Assessment of endogenous ROS production. ROS production was assessed as previously reported (22). In brief, the cell suspension was incubated with 20 mg/liter of 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, Munich, Germany) for 30 min at 35°C at 150 rpm.

FIG 1 Effect of liposomal amphotericin B on *S. cerevisiae* BY4741 and *C. albicans* 596. (A) Study design scheme. (a) Plasma concentration-time curve obtained after a first infusion of AMB-L at 3 mg/liter. (b) The scheme proposed in accordance with AMB-L plasma levels described by Walsh et al (15). (c) Dashed line represents the treatment with a constant concentration of 3 mg/liter AMB-L during 24 h. (B) Viability assessment by CFU enumeration of *S. cerevisiae* BY4741 and *C. albicans* 596 cells exposed to treatment conditions b and c. Data at respective time points are given as means $\pm$ standard deviations. An asterisk indicates significant differences between the two treatment conditions.

Yeast cells were washed ($2,655 \times g$ for 5 min at room temperature; 5417R; Eppendorf) and resuspended in phosphate-buffered saline (PBS; Sigma-Aldrich, Munich, Germany); afterwards, cells were treated as described above. As a control, cells were treated with 0.4 mM H_2O_2 (PanReac, Castellar del Valles, Spain). The FI was determined at FL1 (530 nm). ROS production was calculated by subtracting the FI value of cells treated with simple antifungal agent from that of cells treated with both antifungal agent and DCFH-DA.

TUNEL assay. DNA strand splitting was demonstrated by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) with the *in situ* cell death detection kit, fluorescein, from Roche (Mannheim, Germany). Yeast cells were fixed with 3.7% (vol/vol) formaldehyde (Applchem, Darmstadt, Germany) for 30 min at room temperature and washed thrice with PBS ($2,655 \times g$ for 5 min at room temperature; 5417R; Eppendorf). Afterwards, cell walls were digested with 24 mg/liter of lyticase (Sigma-Aldrich, Munich, Germany) at 37°C for 60 min. Cells were rinsed with PBS, incubated in permeabilization solution (0.1% [vol/vol] Triton X-100 and 0.1% [wt/wt] sodium citrate) for 2 min on ice, and rinsed twice with PBS (5417R; Eppendorf). For a positive control, cells were treated with DNase I enzyme (Roche, Mannheim, Germany) for 10 min and then washed twice in PBS (5417R; Eppendorf). Yeast cells subsequently were incubated with 15 μ l of TUNEL reaction mixture for 60 min at 37°C and then washed twice in PBS (5417R; Eppen-

dorf). Finally, cell suspensions were submitted to flow cytometric analysis, and the percentage of apoptotic cells was determined at FL1 (530 nm).

Simultaneously, 10 μ l of the treated cell suspension was placed in a microscope slide, and the protocol for 4',6-diamidino-2-phenylindole (DAPI; Roche, Mannheim, Germany) nuclear staining was carried out as previously described, using 1 mg/liter of DAPI (23), for further analysis under fluorescence microscopy with a Carl Zeiss Axiovert inverted microscope and laser wavelengths of 405 nm (DAPI) and 488 nm (TUNEL).

Statistical analysis. Results are presented as mean values and the respective standard deviations. A comparison of results was performed using paired-sample Student's *t* test. $P < 0.05$ was considered significant. For calculations of all measures, the SPSS program (version 19.0) was used.

RESULTS

Liposomal amphotericin B effect on cell viability. Our primary goal in this study was to determine the effect of therapeutic plasma concentrations of AMB-L upon cell viability. We focused on AMB-L effects within the first 24 h of exposure, a time period corresponding to the first drug infusion. The effect of AMB-L on yeast cells was evaluated by colony-forming unit count following incubation with decreasing concentrations of AMB-L (Fig. 1B). As

FIG 2 Effect of liposomal amphotericin B on *S. cerevisiae* BY4741 physiological parameters. (A) Cell membrane integrity was assessed with propidium iodide (PI). (B) Cell membrane potential was evaluated using DiBAC₄(3). (C) Metabolic activity was determined by 5-CFDA staining. (D) Endogenous reactive oxygen species (ROS) production as determined by DCFH-DA staining. An asterisk indicates significant differences ($P < 0.05$) between the two treatment conditions.

detailed in Fig. 1B, a slight reduction of viability was observed for up to 6 h of exposure; however, after 6 h of exposure to decreasing concentrations, cells recovered their replication ability, with this effect being most marked after 24 h of exposure (10^6 to 10^9 cells/ml). Conversely, in the case of cells exposed to 3 mg/liter AMB-L for 24 h, a growth reduction was consistently registered throughout the 24-h period, and the cells were unable to replicate. At 24 h, a significant difference between both experimental conditions was registered ($P < 0.001$). Overall, the killing kinetics were similar among all species studied (Table 1). *S. cerevisiae* BY4741 and *C. albicans* 596 are representative examples (Fig. 1B). When comparing such results with the assessment of cell viability with PI (Fig. 2A), we observed some discrepancies. While after 1, 3, and 6 h of incubation with AMB-L under both drug treatment conditions there was a reduction of colony-forming units per milliliter, the cells were not permeable to PI. Only after 24 h was any PI uptake observed, but it was seen in only about 50% of the cells treated with 3 mg/liter AMB-L.

Liposomal amphotericin B-induced alterations on membrane potential. To assess the effect of AMB-L on the cytoplasmic membrane potential of yeast cells, we used DiBAC₄(3) staining.

DiBAC₄(3) enters only depolarized cells, where it binds reversibly to intracellular components, resulting in an increased fluorescent signal. The results obtained regarding membrane depolarization of *S. cerevisiae* BY4741 are depicted in Fig. 2B. Viable nontreated cells stained with DiBAC₄(3) exhibited a small percentage of depolarized cells ($\approx 5\%$), which remained constant over time. Cells treated with AMB-L plasma concentrations displayed an increase of depolarized cells (DC) up to 6 h of incubation; the percentage of DC at 1, 3, and 6 h of incubation was 13.01%, 20.13%, and 45.83%, respectively. Interestingly, after 24 h of incubation, membrane yeast cells seemed to repolarize, corresponding to a DC final value of 19.67%. Conversely, treatment of yeast cells with 3 mg/liter AMB-L resulted in a time-dependent increase of depolarized cells. Figure 2B shows that following 24 h of incubation, cells treated with 3 mg/liter AMB-L displayed higher membrane depolarization than cells treated with plasma concentrations; the percentage of depolarized cells at this time point was 80.45%.

To evaluate whether this mechanism of cell salvage is conserved in the presence of decreasing concentrations of AMB-L during a 24-h period among distinct yeasts, a broad range of yeast species with different phylogenetic relationships (Table 1) were

FIG 3 TUNEL staining of *S. cerevisiae* BY4741 cells exposed to liposomal amphotericin B. (A) Fluorescence microscopy imaging showing TUNEL-positive cells after treatment with AMB-L plasma concentrations (a) and with 3 mg/liter (b) after 3 h, 6 h, and 24 h. (B) Percentage of cells exhibiting damaged DNA (i.e., cells positive by TUNEL) after treatment with AMB-L, as assessed by flow cytometry. An asterisk indicates significant differences ($P < 0.05$) between the two treatment conditions. (C) Nuclear fragmentation as shown by DAPI staining. Fluorescence microscopy imaging with (a) and without (b) a DAPI filter. *S. cerevisiae* cells exposed to AMB-L exhibit an irregular shape and fragmented DNA, two findings in accordance with DNA damage during apoptosis.

assessed by flow cytometry regarding the respective cell membrane potential. The species studied showed different susceptibility profiles to AMB-L with all wild-type *Candida* strains (MIC, ≤ 2 mg/liter), except *C. krusei*, with a MIC of 8 mg/liter. Non-*Candida* yeast genera also showed MIC values of < 2 mg/liter. Regardless of the susceptibility profile, all species studied revealed depolarization of the cell membrane after 6 h of incubation with AMB-L plasma concentrations, followed by repolarization of the cell membrane by 24 h (Table 1). However, cells treated with 3 mg/liter AMB-L exhibited a time-dependent increase in membrane depolarization (data not shown).

Metabolic alterations triggered by AMB-L exposure. To assess metabolic effects of AMB-L on *S. cerevisiae* cells, we performed 5-CFDA staining. 5-CFDA is a cell-permeant esterase substrate; it measures both enzymatic activity and cell membrane integrity. The results obtained for MIF displayed by cells stained with 5-CFDA are detailed in Fig. 2C. The MIF displayed by viable cells not treated with AMB-L following 1 h of treatment was 820.05; it increased after 6 h of incubation (MIF, 1,127.35) and remained constant up to 24 h. After 1 h of incubation, yeast cells treated with plasma concentrations of AMB-L displayed a MIF slightly higher than that of viable cells (901.01). However, following 3 and 6 h, the MIF decreased to values of 721.33 and 549.78, respectively; afterwards, the MIF remained constant until 24 h of treatment. When yeast cells were treated with 3 mg/liter AMB-L

over time, the MIF increased after 1 h of incubation (MIF, 923.54), indicating that the cells were metabolically active. However, after 3 h of incubation, the MIF decreased over time; at 24 h the MIF was 327.49. This event may be related to a lower enzyme activity or cell membrane injury.

Induction of endogenous ROS production by AMB-L. Reactive oxygen species production was assessed by DCFH-DA staining. DCFH-DA is oxidized to highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF) by ROS. Exposure to AMB-L plasma concentrations resulted in reduced ROS formation in *S. cerevisiae* cells ($\approx 18\%$) (Fig. 2D). In contrast, prolonged and constant exposure to 3 mg/liter AMB-L resulted in high formation of ROS ($P < 0.001$); after 6 h of incubation, a significant increase of the number of fluorescent cells (reaching 100%) was registered. This assay reveals that 3 mg/liter AMB-L leads to intracellular accumulation of ROS, which is associated with oxidative damage and possibly is involved in induced programmed cell death.

DNA damage and nuclear fragmentation. DNA fragmentation was measured by TUNEL assay. The percentage of *S. cerevisiae* cells that exhibited TUNEL-positive nuclei after exposure to plasma concentrations of AMB-L for 3 and 6 h was about 20% (Fig. 3). There were significant differences between cells exposed to AMB-L plasma concentrations and to 3 mg/liter AMB-L ($P < 0.001$); following exposure to AMB-L at a constant 3 mg/liter concentration, TUNEL-positive cells increased over time, indicating

apoptotic-like DNA fragmentation; after 24 h of incubation with 3 mg/liter of AMB-L, 60.8% of yeast cells exhibited TUNEL-positive nuclei, twice the proportion of TUNEL-positive cells following exposure to AMB-L plasma concentrations (Fig. 3A and B). During apoptosis, *S. cerevisiae* cells exposed to AMB-L exhibited evidence of nuclear fragmentation associated with DNA damage (Fig. 3C), including irregularly shaped DNA, as previously described (24).

DISCUSSION

Our study clearly demonstrated that exposure to AMB-L plasma concentrations induces compensatory responses at distinct levels, like replicative ability, membrane potential, metabolic activity, endogenous ROS production, and DNA damage. In the treatment regimen with plasma concentrations, yeast cells are exposed to high concentrations of AMB-L (5 to 20 mg/liter) over a short time period (6 h), which can trigger a stress response. Consequently, the yeast cells initially compensate by upregulating their physiological responses to minimize this stress; afterwards, over the remaining 18 h they are exposed to a much lower AMB-L concentration, which allows its recovery. This may explain why the yeast cells exposed to the simulated plasma concentrations are less AMB-L susceptible. Accordingly, no evidence of fungicidal activity was found, which may be responsible for fungal infection persistence in the blood of these patients. Conversely, following constant exposure to 3 mg/liter, cells develop compensatory mechanisms for survival initially; however, as the concentration is kept constant for 24 h, the cells succumb to the drug effects. In addition, in interpreting this data, one also has to consider the fact that most fungal infections localize in the tissues, such as the lungs, kidneys, liver, and spleen. In these tissues, concentrations of AMB-L reach well above the MIC and are maintained at these levels for at least 1 week and longer depending upon the tissues (25, 26).

An important challenge for cell physiology and microorganism survival is a successful, balanced growth when confronted with environmental imbalances. A variety of cellular processes and physiological changes have to be coordinated to allow yeast cells to reproduce, grow, and respond to environmental stresses (27). We demonstrated that yeast cells use distinct time-programmed mechanisms to respond to AMB-L-induced stress. When yeast cells were exposed initially to high concentrations of AMB-L (5 to 20 mg/liter) during the first 6 h, followed by much lower levels of drug, closer to their MIC (simulated plasma concentrations), the loss of replication competency seems to be a relatively early event that could be easily overcome, provided that the yeast then were exposed for longer periods of time to drug concentrations at or below their MICs (Fig. 1B). Previous studies have shown that some yeast cells exposed to amphotericin B demonstrate a capacity for resuscitation, although they are unable to replicate (12, 28). It is notable that increasing and then decreasing AMB-L concentrations rapidly evoke compensatory responses by yeast cells, including the recovery of replication ability. Interestingly, such cells appear to exhibit an intact cell membrane, as shown by PI staining (only about 5% of cells are PI positive after 24 h) (Fig. 2A). Conversely, yeast cells constantly exposed to 3 mg/liter AMB-L lose their viability, as confirmed by colony-forming unit determination, also with clear evidence of cell membrane injury (50% of cells PI positive at 24 h).

Regarding membrane potential, the phenomenon of cell sal-

vage was found to be conserved among different yeasts (pathogenic and nonpathogenic) with different phylogenetic relationships and antifungal susceptibility profiles, suggesting that this recovery is related to insufficient AMB-L exposure. An essential aspect of environmental adaptation is the equilibrium of ion concentration, which determines cell membrane potential (29). It is well known that amphotericin B increases fungal cell membrane permeability to ions (30). Yeast cells initially exposed to high AMB-L concentrations exhibited evidence of plasma membrane depolarization soon after 1, 3, and 6 h of incubation, as shown by DiBAC₄(3) staining; however, in such cells membrane potential was restored afterwards during the time that the cells were exposed to levels of drug close to or below their MICs (Fig. 2B). Our results suggest that with initial exposure to high AMB-L levels, there is a cellular response characterized by recovery of plasma membrane potential which is maintained when the cells then are incubated with low levels of AMB-L (at or below MICs). In contrast, yeast cells exposed to 3 mg/liter AMB-L exhibited a marked time-dependent impairment in membrane potential (Fig. 2B). These results suggest that yeast cell recovery after exposure to high levels of AMB-L is related to subsequent exposure to nonfungicidal concentrations of AMB-L.

Cell metabolic activity was significantly reduced by AMB-L plasma concentrations at 3 and 6 h of incubation, remaining constant for up to 24 h (Fig. 2C). In contrast, yeast cells continuously exposed to AMB-L at constant concentration (3 mg/liter) initially displayed a high metabolic activity after 1 h of incubation, suggesting a different metabolic stress response. This fact is in accordance with the hypothesis that yeast cells exposed to antifungal pressure reprogram their metabolism in response to an environmental stress (31). A previous study involving yeast genome-scale microarrays demonstrated that amino acid metabolism, phosphate metabolism, and carbohydrate metabolism genes are upregulated after treatment with 2.5 mg/liter of amphotericin B (4). After 3, 6, and 24 h of incubation with 3 mg/liter AMB-L, the fluorescence resulting from cFDA cleavage decreased (Fig. 2C); this finding can be explained by membrane pore formation, which may lead to the loss of fluorescence, or by an extremely reduced metabolic activity (32).

Previous studies have shown that amphotericin B induces *C. albicans* apoptosis (13, 14). According to our results, endogenous ROS production was induced following 6 h of AMB-L exposure (Fig. 2D); this finding also was corroborated by results for DNA damage (Fig. 3A and B). Notably, apoptosis was more evident when *S. cerevisiae* was exposed to 3 mg/liter AMB-L than when exposed to AMB-L plasma concentrations, as documented by both types of assays. Interestingly, AMB-L at plasma concentrations did not kill *S. cerevisiae* cells by necrosis; in fact, cells exposed to such concentrations displayed no evidence of plasma membrane damage, as shown by PI staining.

In conclusion, our results using *S. cerevisiae* as a model organism clearly demonstrate that yeast cells can respond in two ways to AMB-L: (i) expression of compensatory responses and survival when, in the plasma, the yeast are initially exposed to high concentrations of AMB-L and then much lower concentrations of the drug for extended periods of time; and (ii) induction of programmed cell death and/or necrosis, occurring at constant high concentrations. These findings provide important insights regarding AMB-L antifungal activity and ultimately may lead to the need to review therapeutic regimens.

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