AAC Journals.ASM.org

Synergistic Effects of Amiodarone and Fluconazole on *Candida tropicalis* Resistant to Fluconazole

Cecília Rocha da Silva,[a,b] João Batista de Andrade Neto,[a] José Júlio Costa Sidrim,[b] Maria Rozzelê Ferreira Ângelo,[c] Hemerson Iury Ferreira Magalhães,[a,d] Bruno Coêlho Cavalcanti,[d] Raimunda Sâmia Nogueira Brilhante,[b] Danielle Silveira Macedo,[d] Manoel Odorico de Moraes,[d] Marina Duarte Pinto Lobo,[e] Thalles Barbosa Grangeiro,[e] Hélio Vitoriano Nobre Júnior[a,b,d]

Department of Clinical and Toxicological Analysis, School of Pharmacy, Laboratory of Bioprospection and Experiments in Yeast (LABEL), Federal University of Ceará, Fortaleza, CE, Brazil[a]; Department of Pathology and Legal Medicine, School of Medicine, Specialized Medical Mycology Center, Federal University of Ceará, Fortaleza, CE, Brazil[b]; Central Public Health Laboratory (LACEN-CE), Fortaleza, CE, Brazil[c]; Department of Physiology and Pharmacology, Federal University of Ceará, Fortaleza, CE, Brazil[d]; Molecular Genetics Laboratory, Department of Biology, Center of Sciences, Federal University of Ceará, Fortaleza, CE, Brazil[e]

There have recently been significant increases in the prevalence of systemic invasive fungal infections. However, the number of antifungal drugs on the market is limited in comparison to the number of available antibacterial drugs. This fact, coupled with the increased frequency of cross-resistance, makes it necessary to develop new therapeutic strategies. Combination drug therapies have become one of the most widely used and effective strategies to alleviate this problem. Amiodarone (AMD) is classically used for the treatment of atrial fibrillation and is the drug of choice for patients with arrhythmia. Recent studies have shown broad antifungal activity of the drug when administered in combination with fluconazole (FLC). In the present study, we induced resistance to fluconazole in six strains of *Candida tropicalis* and evaluated potential synergism between fluconazole and amiodarone. The evaluation of drug interaction was determined by calculating the fractional inhibitory concentration and by performing flow cytometry. We conclude that amiodarone, when administered in combination with fluconazole, exhibits activity against strains of *C. tropicalis* that are resistant to fluconazole, which most likely occurs via changes in the integrity of the yeast cell membrane and the generation of oxidative stress, mitochondrial dysfunction, and DNA damage that could lead to cell death by apoptosis.

In recent decades, there has been an increased incidence of invasive fungal infections (IFIs) in immunocompromised hospitalized patients. These infections have been associated with significant levels of morbidity and mortality and have caused a serious public health problem (1–5). The epidemiology of IFIs is changing, although *Candida albicans* remains the most important fungal agent. However, a notable increase in infections caused by non-*albicans* species (*C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. krusei*) has been reported, and infections by these species account for 36 to 63% of all cases (2, 6–9). These changes in IFI epidemiology can be explained by the high level of resistance of non-*albicans* species to certain antifungal drugs (10).

Fluconazole is one of the most commonly used antifungal agents, and it is used both for prophylaxis and for therapy to combat candidemia. Various mechanisms of resistance to azoles in *Candida* spp. have been described (11, 12). Far fewer antifungal drugs than antibacterial drugs are available on the market, and most of them exhibit fungistatic effects. This fact, together with the increased frequency of cross-resistance, creates greater urgency in the search for new therapeutic strategies (13–15).

Amiodarone (AMD) represents a promising new class of antifungal drug, the class III antiarrhythmics (7), and the drug is viewed as a potential alternative to currently available antifungal therapies. Various studies have demonstrated the fungicidal activity of amiodarone against several species, including a variety of clinically important fungi, such as *C. albicans*, *Cryptococcus neoformans*, *Fusarium oxysporum*, and *Aspergillus nidulans* (17, 18). Additionally, several studies have also demonstrated that the combination of low doses of amiodarone with azoles *in vitro* exhibited a synergistic effect against strains of *C. neoformans*, *C. albicans*, and *A. fumigatus* (7, 19–21).

The aim of the current study was to evaluate and compare the synergistic effect of amiodarone and fluconazole in fluconazole (FLC)-sensitive (clinically isolated in Ceará State, Northeastern Brazil) and FLC-resistant (resistance induced secondarily in the present study) strains of *C. tropicalis* using different standard techniques, such as broth microdilution (BMD) susceptibility methods, flow cytometry procedures, and single-cell gel electrophoresis (comet assay).

MATERIALS AND METHODS

Isolates. We used six strains of *C. tropicalis* that had been isolated from blood samples between 2009 and 2010 at the Central Public Health Laboratory (LACEN-CE) and that were part of the Collection of Yeasts of the Laboratory of Bioprospection and Experiments in Yeast affiliated with the School of Pharmacy at the Federal University of Ceará (LABEL/FF/UFC). The strains were inoculated on Sabouraud dextrose agar (Himedia, Mumbai, India) and incubated at 35°C for 24 h. They were then plated on CHROMagar Candida (Himedia) to assess purity and submitted for molecular identification.

Molecular identification. Genomic DNA was purified using a cetyltrimethylammonium bromide (CTAB)-based protocol, as described previously (18). The nuclear DNA region comprising internal transcribed

Received 10 May 2012 Returned for modification 27 July 2012
Accepted 8 January 2013
Published ahead of print 28 January 2013
Address correspondence to Hélio Vitoriano Nobre Júnior, label_ufc@yahoo.com.br.

doi:10.1128/AAC.00966-12

spacers 1 and 2 (ITS1 and ITS2) and the 5.8S rRNA gene was amplified by PCR using primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GCAAGTAAAAGTCGTAACAAGA-3′), as suggested by White et al. (16). Once the specificity of the amplifications was confirmed, the PCR products from the remaining reactions were purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare Life Sciences, Piscataway, NJ) (23). The concentrations of the purified PCR products were determined by measuring the absorbance of a 10-fold dilution at 260 nm. DNA sequencing was performed using the DYEnamic ET terminator cycle-sequencing kit (GE Healthcare Life Sciences) according to the protocol supplied by the manufacturer, and both strands were sequenced using primers ITS4 and ITS5. The sequencing reactions were then analyzed in a MegaBACE 1000 automatic sequencer (GE Healthcare Bio-Sciences). The parameters for the sequencing runs included injection at 3 kV for 50 s and electrophoresis at 6 kV for 180 min. Automated base calling was performed using Cimarron 3.12 software, and the electropherograms were visualized with Sequence Analyzer v4.0 (Amersham Biosciences, Sunnyvale, CA). The base sequences were then deduced by inspection of each processed data trace, and the complete sequences were assembled using Cap3 software (24). The sequences determined were compared to those previously deposited in the GenBank database using the program BLAST (25).

Development of FLC resistance. A single randomly selected colony from each *C. tropicalis* strain was inoculated into 1 ml of RPMI 1640 (Cultilab, São Paulo, Brazil) and incubated overnight in a rotating drum at 35°C. A 200-μl aliquot of this culture was transferred into 1 ml of fresh culture medium (RPMI 1640) with fluconazole and was further incubated overnight as described above. The following day, an aliquot from each culture was again transferred into fresh medium containing fluconazole and incubated as described. Each day, a 200-μl aliquot from each subculture was mixed with 500 μl of 50% glycerol and frozen at −20°C until testing. In this experiment, the strains were incubated in the presence of fluconazole at a concentration four times the MIC for the compound (26, 27).

Antifungal susceptibility test and evaluation of drug interaction. The BMD susceptibility test was performed according to the document M27-A3 using RPMI broth (pH 7.0) buffered with 0.165 M MOPS (morpholinepropanesulfonic acid) (Sigma Chemical, St. Louis, MO). Fluconazole (Merck Sharp & Dohme, São Paulo, Brazil) and amiodarone were dissolved in distilled water and dimethyl sulfoxide (DMSO) (Sigma Chemical), respectively. Fluconazole was tested in the range of 0.125 to 64 μg/ml and amiodarone in the range of 0.125 to 64 μM. The 96-well culture plates were incubated at 35°C for 48 h, and the results were read visually, as recommended by the CLSI (28). The MIC_{50} was considered to be the concentration that inhibited 50% of fungal growth. The strains were classified as susceptible (S) or resistant (R) to fluconazole according to the following cutoff points: S, MIC ≤ 2 μg/ml; R, MIC ≥ 8 μg/ml (29). The strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as controls (28).

After determining the MIC of each drug, the checkerboard technique was performed. In this technique, each well of the culture plate contained a unique combination of concentrations of the drugs being tested. The percent inhibition of cell growth in the presence of the various drug combinations was determined in relation to the control well containing cells only (30, 31). Thus, the cells were exposed to various concentrations (0.125 to 64 μg/ml) of fluconazole in combination with 0.20 μM amiodarone, and the interaction between fluconazole and amiodarone was determined by calculating the fractional inhibitory concentration index (FICI) as follows: FICI = [FC]/[CFS] + [AC]/[CAS], where [FC] and [AC] represent the MICs of fluconazole and amiodarone acting in combination, whereas [CFS] and [CAS] are the MICs of the same drugs, respectively, acting alone. The interaction between the drugs was classified as synergistic (SYN) (FICI < 0.5), indifferent (IND) (0.5 < FICI ≤ 4.0), or antagonistic (ANT) (FICI > 4.0).

Cell treatments. For the determination of cell density, membrane integrity, mitochondrial transmembrane potential (Δψm), and caspase activation, resistant strains were treated with fluconazole (64 μg/ml), amiodarone (64 μM), or fluconazole (16 μg/ml) plus amiodarone (0.20 μM), and susceptible strains were treated with fluconazole (64 μg/ml) for 4, 6, or 24 h at 35°C. For the evaluation of oxidative stress, DNA damage, and the induction of apoptosis following treatment, the resistant and sensitive strains of *C. tropicalis* were exposed to fluconazole (64 μg/ml), amiodarone (64 μM), or fluconazole (16 μg/ml) plus amiodarone (0.20 μM) for 24 h (11, 32, 33). All tests were performed in triplicate in three independent experiments.

Preparation of yeast suspensions. Cell suspensions were prepared from cultures in the exponential phase of growth. Cells were harvested by centrifugation (1,600 × *g* for 10 min at 4°C), washed twice with 0.85% saline solution (1,200 × *g* for 5 min at 4°C), and then resuspended (~10^6 cells/ml) in HEPES buffer (Sigma Chemical) supplemented with 2% glucose at pH 7.2 (32).

Determination of cell density and membrane integrity. The cell density and membrane integrity of the fungal strains were evaluated by the exclusion of 2 μg/ml propidium iodide (PI). Aliquots removed after 4, 6, and 24 h of incubation with drugs were analyzed by flow cytometry. For each experiment, 10,000 events were evaluated, and cell debris was omitted from the analysis. Cell fluorescence was then determined by flow cytometry using a Guava EasyCyte Mini System cytometer (Guava Technologies, Inc., Hayward, CA) and CytoSoft 4.1 software (34, 35).

Measurement of mitochondrial transmembrane potential. Δψm was determined according to retention of the dye rhodamine 123 (Rho 123) by the fungal strains following 24 h of drug exposure. The cells were washed with phosphate-buffered saline (PBS), incubated with Rho 123 (1 μg/ml) at 35°C for 15 min in the dark, and then washed twice with PBS. Next, the cells were incubated again in PBS at 35°C in the dark for 30 min, and the fluorescence was measured using flow cytometry (Guava EasyCyte Mini System). For each experiment, 10,000 events were evaluated, and cell debris was omitted from the analysis (35, 36).

Efflux of rhodamine 6G. The activity of efflux pumps was determined according to retention of the dye rhodamine 6G (Rho 6G) by the fungal strains following 24 h of drug exposure. Cells were washed with PBS, incubated with Rho 6G (10 μM) and deoxy-D-glucose (5 μM) at 35°C for 2 h, and then washed twice with PBS. The cells were incubated again in PBS at 35°C in the dark for 30 min, and the fluorescence was measured using a multiplate reader (Spectra Count; Packard, Ontario, Canada) at 529 nm for excitation and 553 nm for emission (12).

Detection of ROS. For the detection of reactive oxygen species (ROS) produced over a 24-h culture period, cells were incubated with 20 μM CM-H_2DCFDA [5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester] for 30 min in the dark at 35°C. Then, the cells were harvested, washed, resuspended in PBS, and immediately analyzed by flow cytometry (Guava EasyCyte Mini System). CM-H_2DCFDA readily diffuses through the cell membrane and is hydrolyzed by intracellular esterases to nonfluorescent dichlorofluorescein (DCFH), which is then rapidly oxidized to highly fluorescent DCF (2′,7′-dichlorofluorescein) as a result of a broad range of intracellular oxidative stresses other than H_2O_2 (37). The fluorescence intensity of DCF is proportional to the intracellular amount of ROS formed (38).

Yeast comet assay. The alkaline comet assay was performed essentially as described by Miloshev et al. (39). Up to 200 μl of 0.5% agarose was spread on each slide, and this supportive agarose layer was air dried prior to the application of the cell suspension. Yeast cells were collected by centrifugation in an Eppendorf microcentrifuge for 5 min, washed with distilled water, and resuspended in S buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5). Aliquots of approximately 5 × 10^4 cells were mixed with 0.7% low-melting-point agarose containing 2 mg/ml zymolyase 20T (Seikagaku Corp., Japan) and were spread over the slides. The slides were then covered with coverslips and incubated for 20 min at 30°C. To minimize the activity of endogenous cell enzymes, all further procedures were

TABLE 1 Synergistic effect of FLC and AMD against FLC-resistant strains of *C. tropicalis* isolated in Ceará (MIC_{50} at 48 h)

C. tropicalis strain[a]	MIC_{50}[b]					
	Standard		Combination		FLC-AMD interaction[c]	
	FLC	AMD	FLC	AMD	FICI	Interpretation
1	64	>64	32	0.20	0.50	SYN
2	64	>64	20.16	0.20	0.32	SYN
3	64	>64	20.16	0.20	0.32	SYN
4	64	>64	12.7	0.20	0.20	SYN
5	64	>64	25.4	0.20	0.39	SYN
6	64	>64	12.7	0.20	0.20	SYN

[a] FLC-resistant strains of *C. tropicalis* isolated from biological samples.
[b] The MIC_{50} was defined as the lowest concentration that produced a 50% reduction in growth of fungal cells after 48 h of incubation. The procedure was performed according to CLSI protocol M27-A3. The values are expressed in μg/ml for FLC and in μM for AMD. The MICs represent geometric means of at least three MICs determined on different days.
[c] The synergistic effect of FLC and AMD was calculated based on the FICI (see the text).

performed in a cold room at 8°C to 10°C. The coverslips were removed, and the slides were incubated in 30 mM NaOH, 1 M NaCl, 0.1% laurylsarcosine, 50 mM EDTA (pH 12.3) for 1 h to lyse the spheroplasts. The slides were rinsed three times for 20 min each time in 30 mM NaOH, 10 mM EDTA (pH 12.4) to unwind the DNA, and the slides were then subjected to electrophoresis in the same buffer. Electrophoresis was carried out for 20 min at 0.5 V/cm and 24 mA. After electrophoresis, the gels were neutralized by submerging the slides in 10 mM Tris-HCl, pH 7.5, for 10 min, followed by consecutive 10-min incubations in 76% and 96% ethanol, respectively. Finally, the slides were left to air dry and were stained with ethidium bromide (1 mg/ml) and visualized by fluorescence microscopy (35).

All of the above-mentioned steps were conducted in the dark to prevent additional DNA damage. Images of 100 randomly selected cells (50 cells from each of 2 replicate slides) were analyzed for each experimental group. The cells were scored visually and assigned to one of five classes according to tail size (from undamaged [class 0] to maximally damaged [class 4]), and a damage index value was calculated for each sample of cells. The damage index values, therefore, ranged from 0 (completely undamaged: 100 cells × 0) to 400 (maximum damage: 100 cells × 4) (40). The frequency of tailed cells, which were taken as an indicator of DNA damage, was calculated based on the numbers of cells with tails (DNA strand breaks) and without them.

Analysis of oxidized purine bases in yeast DNA. The levels of oxidized purine bases were estimated using the alkaline comet assay, as described above. Briefly, the slides were removed from the lysis solution and washed three times in enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin [BSA], pH 8.0), drained, and incubated with 70 μl formamidopyrimidine DNA glycosylase (FPG) for 30 min at 35°C. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were visually analyzed per group. The amount of oxidized purines (FPG-sensitive sites) was then determined by subtracting the number of strand breaks (samples incubated with buffer alone) from the total number of breaks obtained after incubation with FPG, according to the methods described previously (9).

Caspase 3/7 activation. Treated and untreated *C. tropicalis* cells were harvested by centrifugation and digested with 2 mg/ml zymolyase 20T (Seikagaku Corp.) in potassium phosphate buffer (PPB) (1 M sorbitol, pH 6.0) for 2 h at 30°C. Caspase 3/7-like activity was determined by flow cytometry using a caspase 3/7 FAM kit from EMD Millipore (Darmstadt, Germany). Yeast cells were incubated with fluorescence-labeled inhibitor of caspases (FLICA) and PI (a necrosis indicator) for 1 h at 35°C in a CO_2 incubator. After incubation, 80 μl of wash buffer was added, and the cells were centrifuged at 5,000 rpm for 5 min. The resulting pellet was resuspended in 200 μl of wash buffer and centrifuged again. The cells were then resuspended in the working solution (PI and wash buffer) and analyzed immediately by flow cytometry (35).

Statistical analysis. The *in vitro* susceptibility experiments and the profiles of synergism and expression were repeated at least three times on different days. Arithmetic means and standard deviations were used to statistically analyze continuous variables (FICI), whereas geometric means were used to compare the MIC results. The data obtained from the flow cytometry experiments and the alkaline comet assay were compared using one-way analysis of variance (ANOVA), followed by the Newman-Keuls test ($P < 0.05$).

RESULTS

Molecular identification. To confirm the identities of the species used in the present work, the complete ITS/5.8S regions (ITS1, 5.8S, and ITS2) of the nuclear ribosomal DNAs from all yeast strains were amplified, sequenced, and compared to sequences deposited in the GenBank database. BLAST searches revealed that the ITS sequences from the six isolates studied here were identical to the ITS/5.8S sequences from different isolates and strains of *C. tropicalis*, and all matching sequences were also from this species (data not shown).

Synergistic effect of amiodarone and fluconazole. Resistance to fluconazole was experimentally induced in the six clinical strains of *C. tropicalis* (Table 1). The development of fluconazole resistance was achieved in approximately 100 days, and the strains were also resistant to amiodarone. However, when these experi-

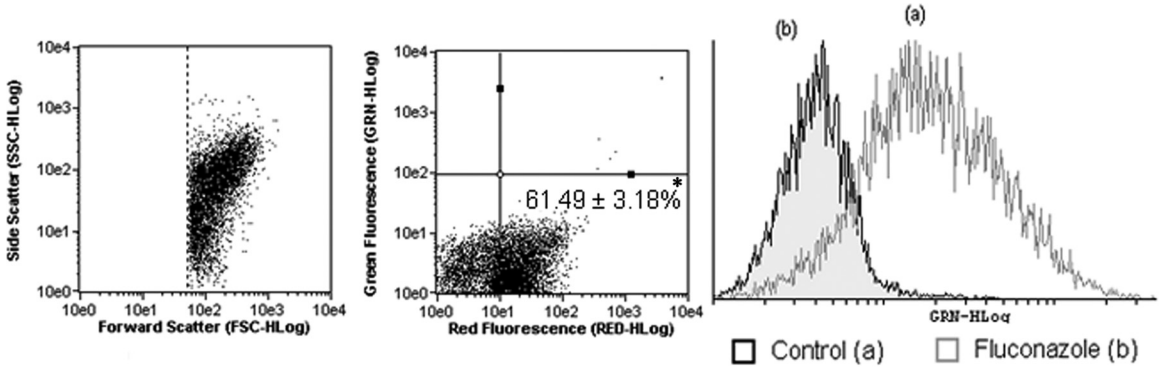

FIG 1 Effect of FLC (64 μg/ml) on an FLC-sensitive strain of *C. tropicalis* after 24 h. (Left) Analysis of changes in cell size/granularity (forward scatter [FSC] × side scatter [SSC]). (Middle) Effect on membrane integrity (as determined by a PI exclusion test). (Right) Effect on Δψm as determined by incorporation of rhodamine 123. *, $P < 0.05$ compared to control (ANOVA and Newman-Keuls test).

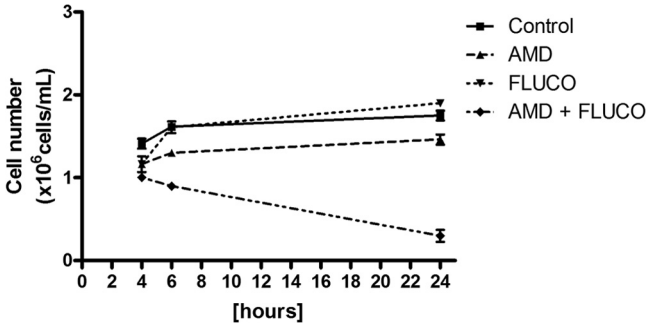

FIG 2 Effects of the different treatments on the viability of FLC-resistant cells of *C. tropicalis* as evaluated by flow cytometry after 24 h. Cells were treated with RPMI (negative control), AMD (64 μM), FLC (FLUCO; 64 μg/ml), and FLC (16 μg/ml) plus AMD (0.20 μM). The data are presented as mean values ± standard errors of the means (SEM) from experiments performed in triplicate.

mentally induced resistant strains were exposed to various concentrations of fluconazole plus 0.20 μM amiodarone, a synergistic effect of the drugs on cell growth was observed (FICI ≤ 0.50) (Table 1). Based on these findings, experiments were devised aimed at elucidating the mechanisms involved in the cytotoxic action of amiodarone and fluconazole against *C. tropicalis*.

Changes in cell size/granularity are synergistically induced by amiodarone and fluconazole in fluconazole-resistant strains. The flow cytometry analysis (side scatter × forward light scatter) of the fluconazole-susceptible strains treated with fluconazole revealed cell shrinkage and nuclear condensation, as evidenced by a decrease in forward light scattering and a transient increase in side scattering (Fig. 1, left). Both of these changes in cell size/granularity observed in fluconazole-sensitive strains are compatible with the presence of dying or dead cells. Regarding fluconazole-resistant *C. tropicalis*, changes in cell size/granularity were not detected in cells subjected to shorter treatments (4 and 6 h) with either drug, alone or in combination (data not shown). On the other hand, all of the fluconazole-resistant *C. tropicalis* strains showed changes in cell size/granularity, which were observed only after 24 h of exposure to both fluconazole and amiodarone (see Fig. 3A).

Loss of cell viability and damage to the plasma membrane are observed after cotreatment of fluconazole-resistant *C. tropicalis* strains with amiodarone and fluconazole. Treatment of fluconazole-sensitive *C. tropicalis* strains with fluconazole caused disruption of the yeast cell membrane (Fig. 1, middle), and this effect was stronger than that found in the fluconazole-resistant strains (see Fig. 3B), as demonstrated by the propidium iodide exclusion assay. As depicted in Fig. 2, the exposure of fluconazole-resistant strains to the azole did not cause reductions in the number of viable cells compared to the control. However, cells treated with fluconazole and amiodarone for 24 h showed a significant decrease in cell viability ($P < 0.05$). Moreover, the plasma membrane stability of fluconazole-resistant cells was not affected after exposure to fluconazole or amiodarone (single treatment), respectively (Fig. 3B). On the other hand, cotreatment (fluconazole plus amiodarone) of the fluconazole-resistant strains induced a significant increase ($P < 0.05$) in the population of cells with lesions in the plasma membrane (Fig. 3B) after 24 h of exposure.

Amiodarone inhibits rhodamine 6G efflux in fluconazole-resistant *C. tropicalis* strains. As shown in Fig. 4, the treatment of fluconazole-resistant strains with fluconazole for 24 h did not cause any significant change ($P < 0.05$) in the efflux of Rho 6G in comparison to the control group (untreated cells). On the other hand, when these fluconazole-resistant strains were treated with amiodarone or amiodarone and fluconazole for 24 h, significant inhibition ($P < 0.05$) in the efflux of Rho 6G was observed, suggesting that amiodarone interferes with the operation of the efflux pump, as described previously (7, 21).

Amiodarone and fluconazole synergistically increase the intracellular production of ROS in fluconazole-resistant *C. tropicalis* strains. A significant increase ($P < 0.05$) in ROS production was observed when fluconazole-sensitive *C. tropicalis* strains were exposed to fluconazole or fluconazole plus amiodarone compared

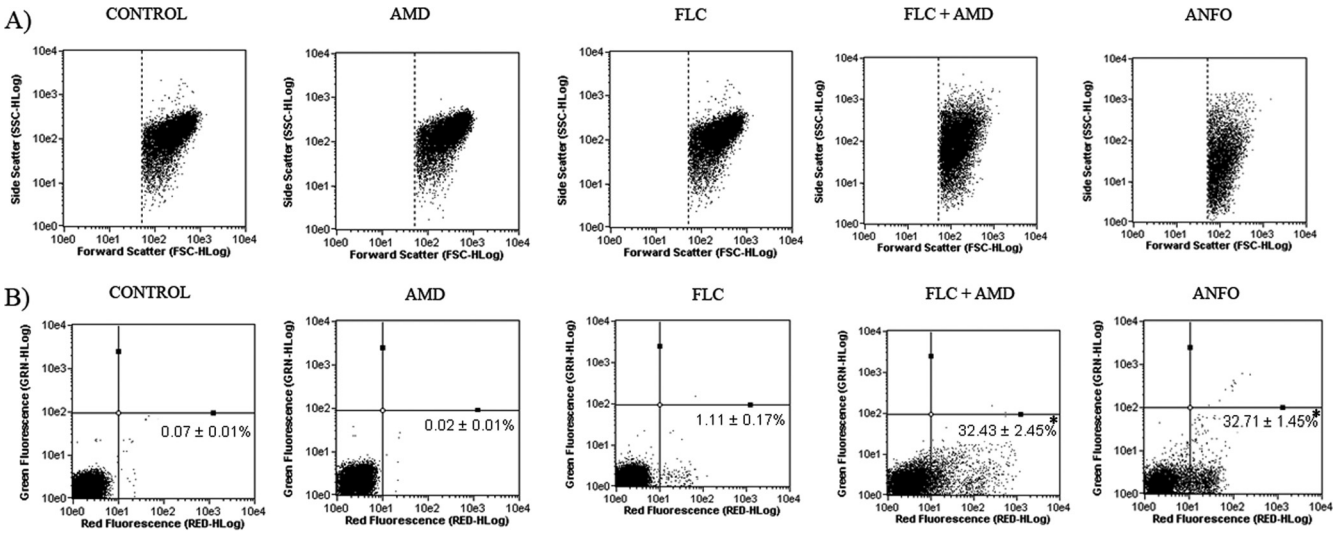

FIG 3 Effects of FLC (64 μg/ml), AMD (64 μM), and FLC (16 μg/ml) plus AMD (0.20 μM) on FLC-resistant strains of *C. tropicalis* after 24 h of exposure. (A) Analysis of changes in cell size/granularity (FSC × SSC). (B) Effect on membrane integrity (as determined by a PI exclusion test). The population of cells in each lower right quadrant corresponds to the percentage of cells with damaged membranes (PI positive). *, $P < 0.05$ compared to the control (ANOVA and Newman-Keuls test). ANFO, anfothericin B.

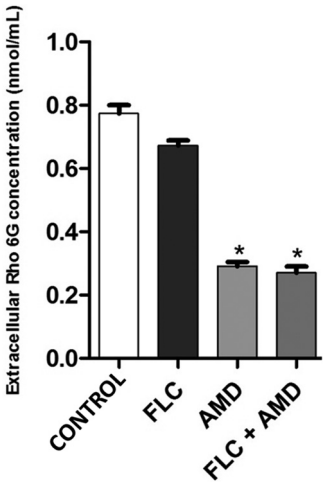

FIG 4 Analysis of Rho 6G uptake and glucose-induced Rho 6G efflux on FLC-resistant strains of *C. tropicalis* exposed to FLC (64 μg/ml), AMD (64 μM), and FLC (16 μg/ml) plus AMD (0.20 μM) for 24 h. Untreated cells were used as a control. *, $P < 0.05$ compared to the control (ANOVA and Newman-Keuls test). The error bars indicate SEM.

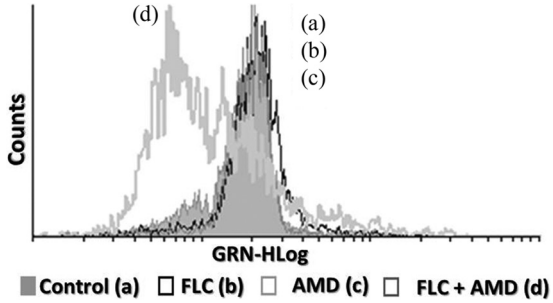

FIG 6 Histograms obtained by flow cytometry analysis of green fluorescence (GRN-HLog) of FLC-resistant *C. tropicalis*. The fluorescence of the cells shows the effects of different treatments on the mitochondrial transmembrane potential in the strains exposed for 24 h to RPMI (a), FLC (64 μg/ml) (b), AMD (64 μM) (c), and FLC (16 μg/ml) plus AMD (0.20 μM) (d).

with the negative-control group (Fig. 5A and B). On the other hand, a significant increase ($P < 0.05$) in ROS production by the fluconazole-resistant strains was observed only in cells treated with both compounds (Fig. 5C and D).

Depolarization of the mitochondrial membrane (Δψm). After 24-h exposure to fluconazole, alterations in the Δψm were observed in the fluconazole-sensitive strains of *C. tropicalis* (Fig. 1, right). In contrast, mitochondrial dysfunctions in fluconazole-resistant strains of *C. tropicalis* were observed only in cells treated with amiodarone plus fluconazole, suggesting that the cotreatment induces a reduction in the mitochondrial transmembrane potential (Fig. 6).

Cotreatment with fluconazole and amiodarone did not induce strand breaks in the genomic DNA of *C. tropicalis*. Strand breaks in genomic DNA were evaluated by a standard alkaline

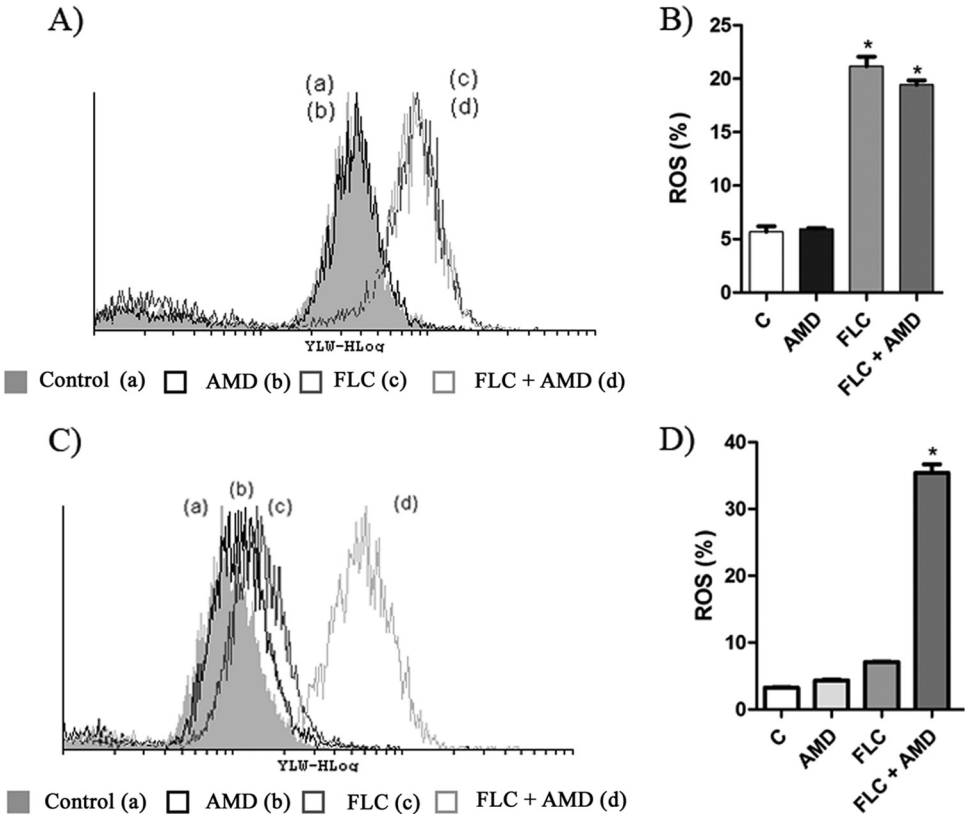

FIG 5 Histograms obtained by flow cytometry analysis of yellow fluorescence (YLW-HLog) of FLC-sensitive (A) and FLC-resistant (C) strains of *C. tropicalis* and percentages of DCF fluorescence-positive cells (ROS production) in FLC-sensitive (B) and FLC-resistant (D) strains exposed to RPMI (a), FLC (64 μg/ml) (b), AMD (64 μM) (c), and FLC (16 μg/ml) plus AMD (0.20 μM) (d) for 24 h. *, $P < 0.05$ compared to the control (lanes C) (ANOVA and Newman-Keuls test). The error bars indicate SEM.

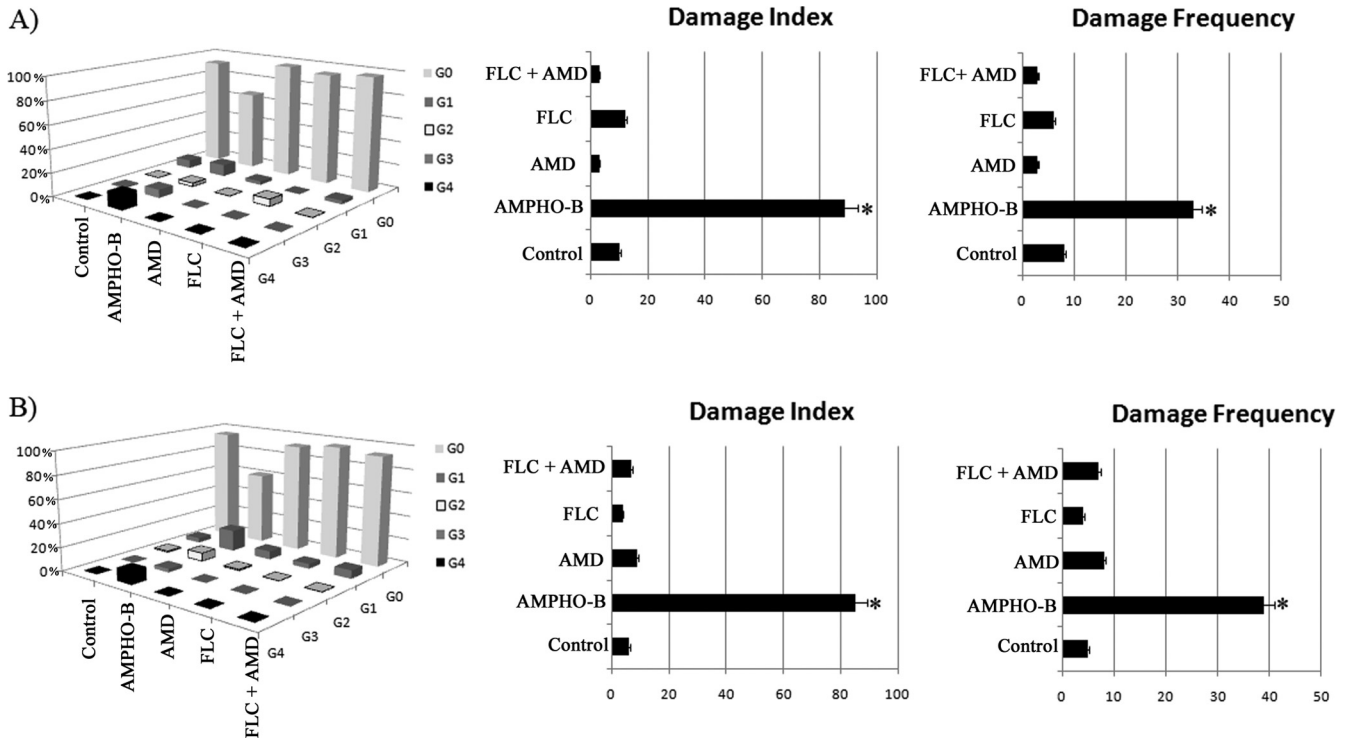

FIG 7 Effects of different treatments on the distribution of damage classes (grades [G]), DNA damage index, and frequency (percent) using the standard alkaline version of the comet assay in *C. tropicalis* strains sensitive (A) and resistant (B) to fluconazole after 24-h exposure. The yeasts were exposed to RPMI, FLC (64 μg/ml), AMD (64 μM), FLC (16 μg/ml) plus AMD (0.20 μM), and amphotericin B (AMPHO-B) (4 μg/ml) as a control. *, $P < 0.05$ compared to the control (ANOVA and Newman-Keuls test). The error bars indicate SEM.

version of the comet assay, and the results were expressed as the damage index (DI) and damage frequency (DF). Thus, the treatment of the *C. tropicalis* strains (sensitive and resistant to fluconazole) with the drugs alone or in combination (fluconazole plus amiodarone) was not able to induce strand breaks in their genomic DNA (Fig. 7A and B) after 24-h exposure compared to untreated cells. On the other hand, the known antifungal agent amphotericin B (4 μg/ml), which was used as a positive control, induced a significant increase in DI and DF compared to untreated cultures of both fluconazole-sensitive and fluconazole-resistant strains of *C. tropicalis* (Fig. 7A and B).

Oxidative DNA damage induced by cotreatment of *C. tropicalis* with amiodarone and fluconazole. As we had observed a pro-oxidative effect of fluconazole, we next decided to evaluate the level of oxidative DNA damage exerted by fluconazole alone or in combination with amiodarone. To this end, we performed the alkaline version of the comet assay using formamidopyrimidine DNA glycosylase (FPG), a base excision repair enzyme that recognizes and removes a wide range of oxidized purines from damaged DNA (41). Figure 8 shows that, following incubation with FPG, there were significant increases in DI and DF in cells from both strains (sensitive and resistant to fluconazole) that were treated with fluconazole alone and strains that were treated with fluconazole plus amiodarone compared to cells not incubated with FPG. Furthermore, amiodarone did not influence the oxidative DNA damage induced by fluconazole. Interestingly, in cultures treated with fluconazole or exposed to fluconazole and amiodarone, the levels of FPG-sensitive sites were more pronounced in the fluconazole-

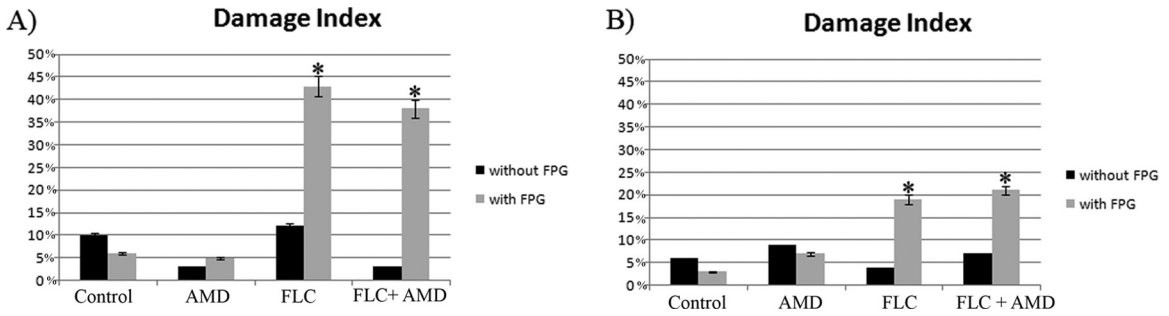

FIG 8 Effects of different treatments on oxidative DNA damage using the modified alkaline version of the comet assay, in the presence or absence of FPG enzyme, in FLC-resistant (A) and FLC-sensitive (B) strains of *C. tropicalis* after 24-h exposure. The yeasts were exposed to RPMI, FLC (64 μg/ml), AMD (64 μM), and FLC (16 μg/ml) plus AMD (0.20 μM). *, $P < 0.05$ compared to the control (ANOVA and Newman-Keuls test). The error bars indicate SEM.

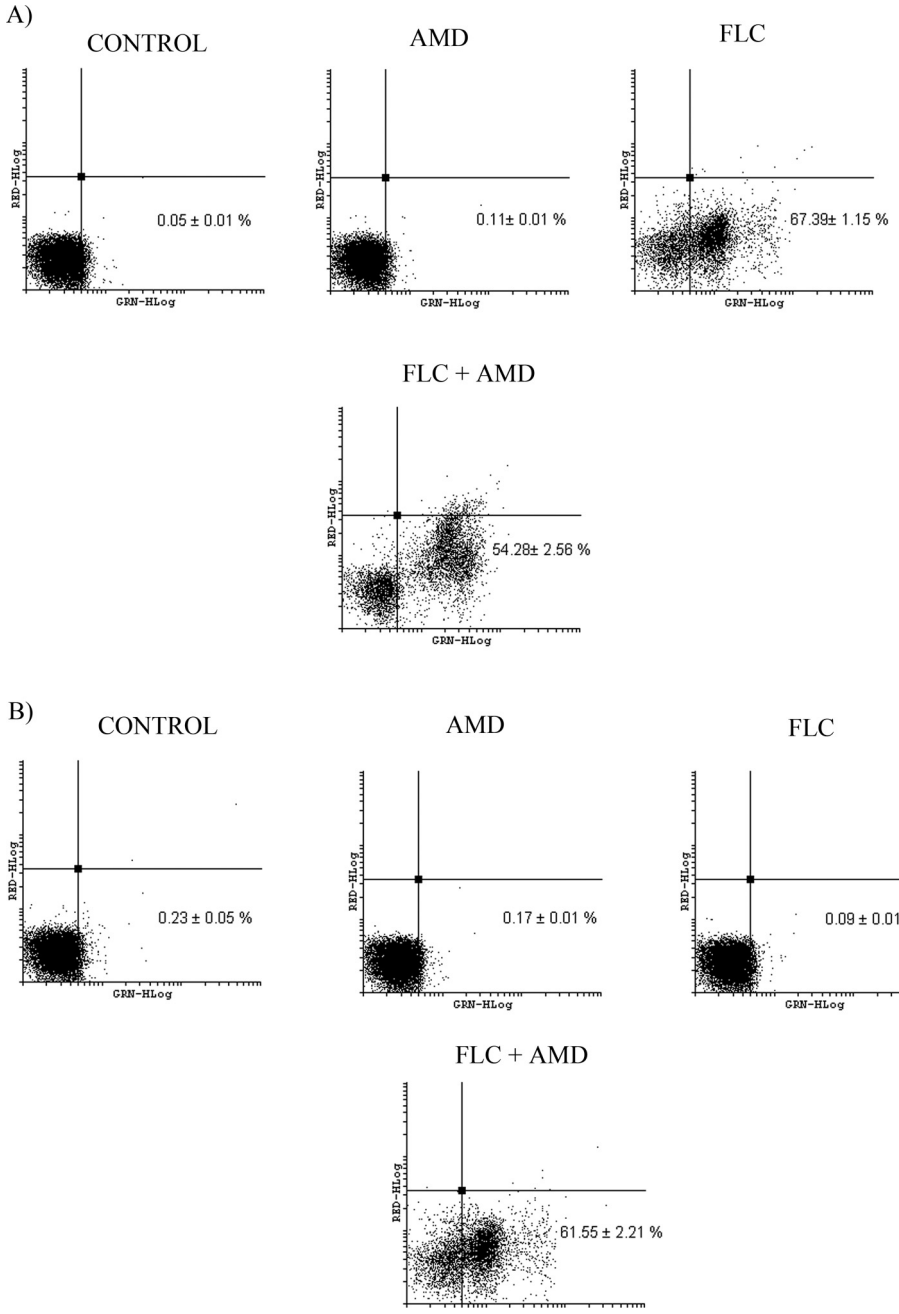

FIG 9 Effects of different treatments on the activation of caspases 3/7 in FLC-sensitive (A) and FLC-resistant (B) *C. tropicalis* strains after 24-h exposure. The yeasts were exposed to RPMI, FLC (64 μg/ml), AMD (64 μM), and FLC (16 μg/ml) plus AMD (0.20 μM). *, $P < 0.05$ compared to the control (ANOVA and Newman-Keuls test).

sensitive strains than in the resistant strains. Moreover, amiodarone did not cause significant differences ($P < 0.05$) in the levels of FPG-sensitive sites that were induced by fluconazole in the genomic DNAs of both strains (sensitive and resistant to fluconazole).

Caspase 3/7 is synergistically activated by amiodarone and fluconazole in fluconazole-resistant *C. tropicalis* strains. In the fluconazole-sensitive strains of *C. tropicalis* (Fig. 9A), an expanded population of caspase-positive cells was detected by flow cytometry following exposure to fluconazole alone (67.39% ± 1.15%) or fluconazole and amiodarone (54.28% ± 2.56%). On the other hand, in the fluconazole-resistant strains, a significant population of caspase-positive cells was detected only when the cells were treated with fluconazole and amiodarone (61.55% ± 2.21%) (Fig. 9B).

DISCUSSION

Non-*albicans* species of *Candida* account for 43.5 to 56.5% of all cases of candidemia (6), and *C. tropicalis* is one of the agents most frequently isolated in episodes of invasive candidiasis, especially in cancer patients (42–45). Studies have reported that the suscepti-

bility of *C. tropicalis* to azoles can be reduced by exposure *in vitro* or *in vivo* to these drugs (26, 46).

In our model, clinical strains of *C. tropicalis* have acquired resistance to fluconazole, but the development of resistance (achieved in ~100 days) was slower than the time frame reported by Barchiesi et al. (26). This difference was likely due to the lower drug concentration used in our protocol.

Moreover, the synergistic effect of amiodarone with azoles against azole-resistant strains of *C. albicans* and *A. fumigatus* has been demonstrated *in vitro* (7, 19, 21). Knorre et al. (47) have reported that the treatment of azole-resistant strains of *Saccharomyces cerevisiae* with amiodarone decreased multidrug resistance due to the inhibition of cellular efflux pumps. In the present study, the synergistic effect of amiodarone and fluconazole was demonstrated using fluconazole-resistant strains of *C. tropicalis* (Table 1).

As shown in Fig. 2, amiodarone plus fluconazole caused cytotoxic effects on fluconazole-resistant strains of *C. tropicalis*, and these changes were not significant in cultures treated with amiodarone or fluconazole alone. Therefore, by blocking efflux pumps, amiodarone likely enhances the function of fluconazole to decrease cell size and increase granularity, as shown in Fig. 3A.

As revealed by the Rho 123 test (Fig. 6), amiodarone plus fluconazole produced mitochondrial dysfunction in fluconazole-resistant strains, suggesting that these drugs affect the mitochondrial respiratory function. In this case, the $\Delta\psi m$ value would be expected to collapse and Rho 123 would likely not accumulate in the mitochondria (36). Studies have shown that amiodarone has the ability to induce a decrease in the mitochondrial transmembrane potential (7, 48). Therefore, according to the results shown in Fig. 2, 3, and 6, treatment of the fluconazole-resistant strains of *C. tropicalis* with amiodarone plus fluconazole altered not only cell size/granularity, but also the $\Delta\psi m$ value. These changes were likely due to the different sites of action of the two drugs (7).

According to Gamarra et al. (7), the synergism between amiodarone and fluconazole can be explained by efflux pump blockage caused by amiodarone. On the other hand, Zhang et al. (49) proposed that fluconazole exacerbated the intracellular levels of Ca^{2+} and H^+ ions, thus interfering with the ionic homeostasis. Ergosterol is the major lipid of fungal cells, and changes in its biosynthetic pathway can alter cellular responses to stress (7, 49).

Our findings also indicate that the synergistic effect of amiodarone and fluconazole involves an increase in the intracellular levels of ROS in the fluconazole-resistant strains of *C. tropicalis*. Other works have reported that the azoles are responsible for endogenous production of ROS in *Candida* spp. (50, 51), and this has been observed in sensitive strains treated with fluconazole.

As shown in Fig. 3B, the cell membrane integrity of *C. tropicalis* strains treated with fluconazole plus amiodarone was clearly altered, as evaluated by the incorporation of PI, and this finding was likely due to the severe membrane damage caused by increased levels of ROS.

Yeast cells exhibit a wide range of dose-dependent responses to increasing ROS concentrations (52–54). At higher doses, cell death occurs in a fraction of cells and results in the acquisition of phenotypes characteristic of caspase-dependent apoptosis (55).

Extensive DNA fragmentation occurs frequently in the early stages of apoptosis, and it is an irreversible step that leads to cell death (52, 56). The alkaline version of the comet assay (standard protocol) is a sensitive procedure used to quantify the different types of DNA damage in cells, including alkali-labile sites, single-stranded breaks, and double-stranded breaks (57). Our data revealed that exposure of *C. tropicalis* strains to fluconazole plus amiodarone (Fig. 7) did not induce any significant increase in the DNA migration pattern (DNA fragments). However, the standard comet assay did reveal a significant increase in DNA breaks in *C. tropicalis* strains treated with amphotericin B (4 μg/ml), which was used as a positive control.

Due to these results, the alkaline version of the comet assay was conducted in the presence of FPG to verify oxidative damage to the DNA (9, 58, 59). The results shown in Fig. 8 indicate that significant DNA damage was caused by the fluconazole-plus-amiodarone treatment, and these results are expressed as the index of DNA damage after treatment with the DNA repair enzyme (FPG). Moreover, the results obtained after incubation with FPG clearly show that DNA migration was increased in the treated strains of *C. tropicalis*, and the extent of oxidative DNA damage caused by the synergism between fluconazole and amiodarone was significantly higher. This increase was likely due to the ability of FPG to recognize purines (adenine and guanine) within DNA that were oxidized by the ROS produced following fluconazole-plus-amiodarone treatment (Fig. 5). Based on such results, we suggest that the synergistic effect of fluconazole plus amiodarone facilitates oxidative damage to DNA via ROS formation.

These results prompted us to perform further study on the physiological state of the cells, particularly with regard to the occurrence of apoptosis and/or necrosis induced by fluconazole plus amiodarone via caspase 3/7. As shown in Fig. 9, an increase in the number of apoptotic cells was observed in the fluconazole-resistant strains treated with fluconazole plus amiodarone (61.55% ± 2.21%).

Conclusion. In conclusion, treatment with fluconazole plus amiodarone caused moderate *in vitro* cytotoxicity in fluconazole-resistant strains of *C. tropicalis.* Although this treatment altered the integrity of the plasma and mitochondrial membranes, as described above, the synergism between fluconazole and amiodarone also seemed to cause DNA damage, leading to apoptotic cell death.

ACKNOWLEDGMENTS

This research was supported by grants and fellowships from CNPq, CAPES/Brazil, and FUNCAP/Ceará.

We declare no conflicts of interest concerning this article.

REFERENCES

1. **Horn D, Fishman J, Steinbach W, Anaissie E, Marr K, Olyaei A, Pfaller M, Weiss M, Webster K, Neofytos D.** 2007. Presentation of the PATH Alliance registry for prospective data collection and analysis of the epidemiology, therapy, and outcomes of invasive fungal infections. Diagn. Microbiol. Infect. Dis. **59**:407–414.
2. **Horn DL, Neofytos D, Anaissie EJ, Fishman JA, Steinbach WJ, Olyaei AJ, Marr KA, Pfaller MA, Chang CH, Webster KM.** 2009. Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. Clin. Infect. Dis. **48**:1695–1703.
3. **Pfaller MA, Castanheira M, Diekema DJ, Messer SA, Jones RN.** 2011. Wild-type MIC distributions and epidemiologic cutoff values for fluconazole, posaconazole, and voriconazole when testing *Cryptococcus neoformans* as determined by the CLSI broth microdilution method. Diagn. Microbiol. Infect. Dis. **71**:252–259.
4. **Pfaller MA, Diekema DJ.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. **20**:133–163.
5. **Sellami A, Sellami H, Neji S, Makni F, Abbes S, Cheikhrouhou F, Chelly H, Bouaziz M, Hammami B, Ben Jemaa M, Khaled S, Ayadi A.** 2011. Antifungal susceptibility of bloodstream *Candida* isolates in Sfax hospital, Tunisia. Mycopathologia **171**:417–422.

6. **Chi HW, Yang YS, Shang ST, Chen KH, Yeh KM, Chang FY, Lin JC.** 2011. *Candida albicans* versus non-*albicans* bloodstream infections: the comparison of risk factors and outcome. J. Microbiol. Immunol. Infect. **44**:369–375.
7. **Gamarra S, Rocha EM, Zhang YQ, Park S, Rao R, Perlin DS.** 2010. Mechanism of the synergistic effect of amiodarone and fluconazole in *Candida albicans*. Antimicrob. Agents Chemother. **54**:1753–1761.
8. **Graybill JR, Najvar LK, Holmberg JD, Luther MF.** 1995. Fluconazole, D0870, and flucytosine treatment of disseminated *Candida tropicalis* infections in mice. Antimicrob. Agents Chemother. **39**:924–929.
9. **da Silva EN, Jr, Cavalcanti BC, Guimaraes TT, Pinto Mdo C, Cabral IO, Pessoa C, Costa-Lotufo LV, de Moraes MO, de Andrade CK, Dos Santos MR, de Simone CA, Goulart MO, Pinto AV.** 2011. Synthesis and evaluation of quinonoid compounds against tumor cell lines. Eur. J. Med. Chem. **46**:399–410.
10. **Gonzalez GM, Elizondo M, Ayala J.** 2008. Trends in species distribution and susceptibility of bloodstream isolates of *Candida* collected in Monterrey, Mexico, to seven antifungal agents: results of a 3-year (2004 to 2007) surveillance study. J. Clin. Microbiol. **46**:2902–2905.
11. **Pina-Vaz C, Rodrigues AG, Costa-de-Oliveira S, Ricardo E, Mardh PA.** 2005. Potent synergic effect between ibuprofen and azoles on *Candida* resulting from blockade of efflux pumps as determined by FUN-1 staining and flow cytometry. J. Antimicrob. Chemother. **56**:678–685.
12. **Vandeputte P, Larcher G, Bergès T, Renier G, Dominique C, Bouchara JP.** 2005. Mechanisms of azole resistance in a clinical isolate of *Candida tropicalis*. Antimicrob. Agents Chemother. **49**:4608–4615.
13. **Pina-Vaz C, Sansonetty F, Rodrigues AG, Costa-de-Oliveira S, Martinez-de-Oliveira J, Fonseca AF.** 2001. Susceptibility to fluconazole of *Candida* clinical isolates determined by FUN-1 staining with flow cytometry and epifluorescence microscopy. J. Med. Microbiol. **50**:375–382.
14. **Tobudic S, Kratzer C, Presterl E.** 2012. Azole-resistant *Candida* spp.—emerging pathogens? Mycoses **55**:24–32.
15. **Vanden Bossche H, Warnock DW, Dupont B, Kerridge D, Sen Gupta S, Improvisi L, Marichal P, Odds FC, Provost F, Ronin O.** 1994. Mechanisms and clinical impact of antifungal drug resistance. J. Med. Vet. Mycol. **32**(Suppl. 1):189–202.
16. **White T, Bruns T, Lee S, Taylor J.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p 315–322. *In* Innis MA, Gefland DH, Sninsky JJ, White TJ (ed), PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA.
17. **Courchesne WE.** 2002. Characterization of a novel, broad-based fungicidal activity for the antiarrhythmic drug amiodarone. J. Pharmacol. Exp. Ther. **300**:195–199.
18. **Wakiec R, Prasad R, Morschhauser J, Barchiesi F, Borowski E, Milewski S.** 2007. Voriconazole and multidrug resistance in *Candida albicans*. Mycoses **50**:109–115.
19. **Afeltra J, Vitale RG, Mouton JW, Verweij PE.** 2004. Potent synergistic in vitro interaction between nonantimicrobial membrane-active compounds and itraconazole against clinical isolates of *Aspergillus fumigatus* resistant to itraconazole. Antimicrob. Agents Chemother. **48**:1335–1343.
20. **Gupta SS, Ton VK, Beaudry V, Rulli S, Cunningham K, Rao R.** 2003. Antifungal activity of amiodarone is mediated by disruption of calcium homeostasis. J. Biol. Chem. **278**:28831–28839.
21. **Guo Q, Sun S, Li Y, Yu J, Shi C.** 2008. Synergistic activity of azoles with amiodarone against clinically resistant *Candida albicans* tested by chequerboard and time-kill methods. J. Med. Microbiol. **57**:457–462.
22. Reference deleted.
23. **Sambrook J, Fritsch EF, Maniatis T.** 1989. The condensed protocols from Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. **Huang X, Madan A.** 1999. CAP3: a DNA sequence assembly program. Genome Res. **9**:868–877.
25. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
26. **Barchiesi F, Calabrese D, Sanglard D, Falconi Di Francesco L, Caselli F, Giannini D, Giacometti A, Gavaudan S, Scalise G.** 2000. Experimental induction of fluconazole resistance in *Candida tropicalis* ATCC 750. Antimicrob. Agents Chemother. **44**:1578–1584.
27. **Pinto e Silva AT, Costa-de-Oliveira S, Silva-Dias A, Pina-Vaz C, Rodrigues AG.** 2009. Dynamics of in vitro acquisition of resistance by *Candida parapsilosis* to different azoles. FEMS Yeast Res. **9**:626–633.
28. **Clinical and Laboratory Standards Institute.** 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A3, 3rd ed. Clinical and Laboratory Standards Institute, Wayne, PA.
29. **Pfaller MA, Diekema DJ.** 2012. Progress in antifungal susceptibility testing of *Candida* spp. by use of Clinical and Laboratory Standards Institute broth microdilution methods, 2010 to 2012. J. Clin. Microbiol. **50**:2846–2856.
30. **Endo E.** 2007. Synergistic effect of the crude extract and fractions of *Punica granatum* against *Candida albicans* and synergy with fluconazole. Ph.D. dissertation. State University of Maringá, Maringá, Brazil.
31. **Odds FC.** 2003. Synergy, antagonism, and what the chequerboard puts between them. J. Antimicrob. Chemother. **52**:1.
32. **Pina-Vaz C, Rodrigues AG.** 2010. Evaluation of antifungal susceptibility using flow cytometry. Methods Mol. Biol. **638**:281–289.
33. **Rudensky B, Broide E, Berko N, Wiener-Well Y, Yinnon AM, Raveh D.** 2008. Direct fluconazole susceptibility testing of positive *Candida* blood cultures by flow cytometry. Mycoses **51**:200–204.
34. **Joung YH, Kim HR, Lee MK, Park AJ.** 2007. Fluconazole susceptibility testing of *Candida* species by flow cytometry. J. Infect. **54**:504–508.
35. **Pinkerton DM, Banwell MG, Garson MJ, Kumar N, Moraes MO, Cavalcanti BC, Barros FWA, Pessoa C.** 2010. Antimicrobial and cytotoxic activities of synthetically derived tambjamines C and E–J, BE-18591, and a related alkaloid from the marine bacterium *Pseudoalteromonas tunicate*. Chem. Biodivers. **7**:1311–1324.
36. **Ludovico P, Sansonetty F, Corte-Real M.** 2001. Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry. Microbiology **147**:3335–3343.
37. **Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM.** 1999. Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2′,7′-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. Free Radic. Biol. Med. **27**:146–159.
38. **LeBel CP, Ischiropoulos H, Bondy SC.** 1992. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. **5**:227–231.
39. **Miloshev G, Mihaylov I, Anachkova B.** 2002. Application of the single cell gel electrophoresis on yeast cells. Mutat. Res. **513**:69–74.
40. **Collins AR.** 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol. Biotechnol. **26**:249–261.
41. **Serre L, Jesus KP, Boiteux S, Zelwer C, Castaing B.** 2002. Crystal structure of the *Lactococcus lactis* formamidopyrimidine-DNA glycosylase bound to an abasic site analogue-containing DNA. EMBO J. **21**:2854–2865.
42. **Almirante B, Rodriguez D, Park BJ, Cuenca-Estrella M, Planes AM, Almela M, Mensa J, Sanchez F, Ayats J, Gimenez M, Saballs P, Fridkin SK, Morgan J, Rodriguez-Tudela JL, Warnock DW, Pahissa A.** 2005. Epidemiology and predictors of mortality in cases of *Candida* bloodstream infection: results from population-based surveillance, Barcelona, Spain, from 2002 to 2003. J. Clin. Microbiol. **43**:1829–1835.
43. **Kucukates E, Erturan Z, Susever S, Yegenoglu Y.** 2005. In vitro susceptibility of yeasts isolated from patients in intensive care units to fluconazole and amphotericin B during a 3-year period. APMIS **113**:278–283.
44. **Wingard JR.** 1995. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. Clin. Infect. Dis. **20**:115–125.
45. **Wingard JR, Merz WG, Saral R.** 1979. *Candida tropicalis*: a major pathogen in immunocompromised patients. Ann. Intern. Med. **91**:539–543.
46. **Barchiesi F, Arzeni D, Del Prete MS, Sinicco A, Falconi Di Francesco L, Pasticci MB, Lamura L, Nuzzo MM, Burzacchini F, Coppola S, Chiodo F, Scalise G.** 1998. Fluconazole susceptibility and strain variation of *Candida albicans* isolates from HIV-infected patients with oropharyngeal candidosis. J. Antimicrob. Chemother. **41**:541–548.
47. **Knorre DA, Krivonosova TN, Markova OV, Severin FF.** 2009. Amiodarone inhibits multiple drug resistance in yeast *Saccharomyces cerevisiae*. Arch. Microbiol. **191**:675–679.
48. **Maresova L, Muend S, Zhang YQ, Sychrova H, Rao R.** 2009. Membrane hyperpolarization drives cation influx and fungicidal activity of amiodarone. J. Biol. Chem. **284**:2795–2802.
49. **Zhang Y-Q, Gamarra S, Garcia-Effron G, Park S, Perlin DS, Rao R.** 2010. Requirement for ergosterol in V-ATPase function underlies antifungal activity of azole drugs. PLoS Pathog. **6**:e1000939. doi:10.1371/journal.ppat.1000939.

50. **Kobayashi D, Kondo K, Uehara N, Otokozawa S, Tsuji N, Yagihashi A, Watanabe N.** 2002. Endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. Antimicrob. Agents Chemother. **46**:3113–3117.
51. **Rogers PD, Vermitsky JP, Edlind TD, Hilliard GM.** 2006. Proteomic analysis of experimentally induced azole resistance in *Candida glabrata*. J. Antimicrob. Chemother. **58**:434–438.
52. **Guerreiro-Salvador V.** 2009. Assessment of Apoptosis and Necrosis in *Saccharomyces cerevisiae* in Fermentation Vinárias. Ph.D. dissertation. University of Minho, Braga, Portugal.
53. **Perrone GG, Tan SX, Dawes IW.** 2008. Reactive oxygen species and yeast apoptosis. Biochim. Biophys. Acta **1783**:1354–1368.
54. **Temple MD, Perrone GG, Dawes IW.** 2005. Complex cellular responses to reactive oxygen species. Trends Cell Biol. **15**:319–326.
55. **Madeo F, Herker E, Maldener C, Wissing S, Lachelt S, Herlan M, Fehr M, Lauber K, Sigrist SJ, Wesselborg S, Frohlich KU.** 2002. A caspase-related protease regulates apoptosis in yeast. Mol. Cell **9**:911–917.
56. **Ribeiro GF, Corte-Real M, Johansson B.** 2006. Characterization of DNA damage in yeast apoptosis induced by hydrogen peroxide, acetic acid, and hyperosmotic shock. Mol. Biol. Cell **17**:4584–4591.
57. **Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M, Sasaki YF, Thybaud V, Uno Y, Vasquez M, Hartmann A.** 2007. Fourth International Workgroup on Genotoxicity Testing: results of the in vivo Comet assay workgroup. Mutat. Res. **627**:31–35.
58. **Cavalcanti BC, Barros FW, Cabral IO, Ferreira JR, Magalhaes HI, Junior HV, da Silva Junior EN, de Abreu FC, Costa CO, Goulart MO, Moraes MO, Pessoa C.** 2011. Preclinical genotoxicology of nor-beta-lapachone in human cultured lymphocytes and Chinese hamster lung fibroblasts. Chem. Res. Toxicol. **24**:1560–1574.
59. **Cavalcanti BC, Bezerra DP, Magalhaes HI, Moraes MO, Lima MA, Silveira ER, Camara CA, Rao VS, Pessoa C, Costa-Lotufo LV.** 2009. Kauren-19-oic acid induces DNA damage followed by apoptosis in human leukemia cells. J. Appl. Toxicol. **29**:560–568.