The antiarrhythmic drug amiodarone has been found to have fungicidal activity. In *Saccharomyces cerevisiae*, its antifungal activity is mediated by calcium overload stress, which leads to a rapid nuclear accumulation of the calcineurin-regulated transcription factor *CRZ1*. In addition, low doses of amiodarone have been reported to be synergistic with fluconazole in fluconazole-resistant *Candida albicans*. To establish its mechanism of toxicity in *C. albicans*, we used expression profiling of key pathway genes to examine cellular responses to amiodarone alone and in combination with fluconazole. Gene expression profiling of 59 genes was done in five *C. albicans* strains (three fluconazole-susceptible strains and two fluconazole-resistant strains) after amiodarone and/or fluconazole exposure. Of the 59 genes, 27 analyzed showed a significant change (>2-fold) in expression levels after amiodarone exposure. The up- or downregulated genes included genes involved in Ca\(^{2+}\) homeostasis, cell wall synthesis, vacuolar/lysosomal transport, diverse pathway regulation, stress response, and pseudohyphal morphogenesis. As expected, fluconazole induces an increase in ergosterol pathway genes expression levels. The combination treatment significantly dampened the transcriptional response to either drug, suggesting that synergism was due to an inhibition of compensatory response pathways. This dampening resulted in a decrease in total ergosterol levels and decreased pseudohyphal formation, a finding consistent with decreased virulence in a murine candidiasis model.

*Candida albicans* is the most frequently observed opportunistic human fungal pathogen causing mucosal and systemic infections in individuals with compromised immune defenses (44). Antifungal therapy is limited by the paucity of chemical classes, toxicity, drug resistance, moderate response rates, and substantial interpatient variation in serum drug levels. Thus, candidiasis remains a challenging opportunistic infection with high mortality, despite current available treatment. There is a pressing need for alternative treatments with new drug classes representing novel drug targets. One promising new antifungal class is represented by amiodarone (AMD), a drug now in clinical use as an antiarrhythmic. AMD has shown fungicidal activity against yeasts and a range of clinically important fungi, including *C. albicans*, Cryptococcus neoformans, Fusarium oxysporum, and Aspergillus nidulans (9, 53). In addition, low doses of AMD have been reported to be synergistic with different azoles in itraconazole-resistant *A. fumigatus* strains (1) and also in the protozoans *Trypanosoma cruzi* (4) and Leishmania mexicana (49).

In *Saccharomyces cerevisiae*, it is known that AMD affects calcium homeostasis (10), leading to an immediate influx of Ca\(^{2+}\) and a rapid activation of the calcineurin pathway, including nuclear accumulation of the calcineurin-regulated *Crl2p*. Transcriptional profiling also revealed an apparent disruption of nutrient sensing/signaling within minutes based on the up-regulation of genes involved in utilization of alternative carbon and nitrogen sources and in mobilizing energy reserves in a Ca\(^{2+}\)-independent fashion (53). Genes involved in all stages of the cell cycle were downregulated by AMD, and a prominent calcineurin-dependent delay in G\(_2\)/M transition was observed (18, 53). Collectively, these data suggest that AMD influences a set of cellular pathways distinct from existing antifungal drugs.

Less is known about the mechanism of action of AMD in *C. albicans*. As a first approximation, it can be inferred that AMD acts in the same way as in *S. cerevisiae*, inducing calcium stress and modifying the calcineurin pathway regulation altering the tolerance to antifungal agents, cation homeostasis, and virulence (47). However, the direct and indirect components of the calcineurin pathway still remain to be elucidated in this yeast species. Moreover, the mechanism of regulation differs between fungal species (21), which makes it critical to evaluate AMD action in pathogenic fungi.

Recently, it was observed that synergy occurred with the combination fluconazole (FLC) and AMD against FLC-resistant *C. albicans* (17). This unexpected result raised the possibility of a novel pathway, perhaps influenced by changes in membrane composition, which contributes to the observed synergy. To evaluate the underlying basis of AMD toxicity in *C. albicans*, expression profiling of key pathway genes, some identified previously in *S. cerevisiae*, was used to examine cellular responses to AMD and AMD-FLC treatment. These observations were confirmed and extended by analysis of total ergosterol content, *in vitro* hyphal growth in liquid media and a murine candidiasis model.
was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma). AMD (Sigma-Aldrich, St. Louis, MO) cation, and filamentous forms inhibition studies. Antifungal agents utilized were FLC (Pfizer, New York, NY) and AMD-susceptible strain 610 and showed genic with the FLC-susceptible strain 610 and control strains ATCC 90028 and SC5314 were used only for MIC and synergism evaluation, ergosterol quantification (kindly provided by Richard D. Cannon) (20). Control strains ATCC 90028 and SC5314 were used throughout the MIC of each drug in combination with the other. These MIC combinations together with the standard MICs were used to obtain the FIC values displayed in the table.

### MATERIALS AND METHODS

#### Strains and compounds.

Seven C. albicans strains were used throughout the present study, including five FLC-susceptible strains (clinical strains 1002 and 610 and control strains ATCC 36082, ATCC 90028, and SC5314), and two FLC-resistant strains (Table 1). Strain 3795 is an FLC-resistant isolate harboring two ERG11 mutations (T376C and A249G) and is isogenic with the FLC-susceptible strain 1002 (42). The other FLC-resistant strain (strain 611) is isogenic with the FLC-susceptible strain 610 and showed CDR1 overexpression (kindly provided by Richard D. Cannon) (20). Control strains ATCC 90028 and SC5314 were used only for MIC and synergism evaluation, ergosterol quantification, and filamentous forms inhibition studies. Antifungal agents utilized were FLC (Pfizer, New York, NY) and AMD (Sigma-Aldrich, St. Louis, MO). AMD was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma).

#### Antifungal susceptibility testing and drug interaction evaluation.

AMD activity varies depending on the pH (37). Thus, the working pH was established in order to obtain the biggest AMD effect. Individual MICs and minimum fungicidal concentrations (MFCs) were obtained for all of the strains according to the CLSI document M27-A3 (6) using RPMI 1640 medium buffered with morpholinepropanesulfonic acid at different pH (ranging from pH 4 to 8) (33, 53). AMD-FLC in vitro drug interactions were evaluated with a two-dimensional, two-antigen broth microdilution checkerboard technique using the fractional inhibitory concentration (FIC) (1, 39).

#### Establishing the optimal culture conditions for expression profiling.

ATCC 36082 strain was grown in YPD agar (1% yeast extract, 2% Bacto peptone, 2% dextrose, 1.5% agar) for 16 h at 37°C. Then, one colony was transferred to YPD broth and incubated at 37°C for 24 h at 250 rpm. Subsequently, the culture was harvested and washed two times with sterile distilled water. From this pellet, cells were resuspended in RPMI 1640 pH 5 in the presence of different drug concentrations (AMD from 0.5 to 25 μg/mL and FLC from one-half MIC to full MIC) and different exposure times (5, 10, 15, 30, 45, 60, and 90 min). DMSO was added to the control cultures in order to approximate a normal distribution, the MICs were transformed to log2 values to establish susceptibility differences between strains. Both on-scale and off-scale results were included in the analysis. The off-scale MICs were considered as the MIC plus two dilutions.

#### RNA isolation and expression profiling.

A 10^7-CFU/ml inoculum was obtained as described above. Cells were grown in RPMI 1640 pH 5 in the presence of 25 μM AMD for 10 min and/or one-half MIC of FLC for 1 h. Total RNA was extracted by a hot phenol-chloroform extraction and ethanol precipitation protocol (7). Gene expression profiles were performed using a one-step Sybr green quantitative reverse transcription-PCR kit (Strategene, La Jolla, CA) on a Strategene Mx3005P multiplex quantitative PCR system. C. albicans URA3 (GenBank accession no. XP_721787.1) was used for normalization. The relative expression was evaluated using the method described by Pfaffl (43). Differential expression was analyzed for 59 C. albicans genes in the following functional categories: Ca^2^+-pumps/channels/transporters (n = 6), calmodulin-calcinurin pathway (n = 4), cell wall (n = 7), alkaline pH and cation overload response (n = 2), GTPase activity (n = 2), transcription factor activity (n = 6), cell cycle (n = 3), heat shock protein (n = 1), oxidation of fatty acids (n = 2), ergosterol biosynthesis (n = 7), morphogenesis and hyphal formation (n = 5), Tor signaling pathway (n = 3), GDP-mannose transporter (n = 1), amino acid transport and metabolism (n = 2), ammonium transmembrane transporters (n = 2), and glucose metabolism and transporters (n = 6) (see the supplemental material).

#### Esterol extraction and quantification.

Sterols were extracted by the alcoholic KOH method and ergosterol content was established as a percentage of the dry weight of the cells by the method described by Arthington-Skaggs et al., with modifications (3). Inocula of 10^7 CFU/ml were obtained as described above and inoculated into 100 ml of RPMI 1640 (pH 5) alone or RPMI 1640 (pH 5) plus AMD, FLC, or AMD-FLC. Then, they were incubated for 16 h at 37°C at 250 rpm. Finally, the cells were divided in two fractions and weighed. The first fraction was used for moisture determination by drying the cells at 50°C until achieving a constant weight. The second fraction was used to measure the ergosterol content of the samples by scanning spectrophotometrically between 240 and 300 nm with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) (3).

In vitro study. In vivo studies were carried out in a murine candidiasis model. Female BALB/c mice (CRL) were challenged with the C. albicans strains 1002, 3795, 610, and 611 administered by tail vein injection. At 3 h postinfection, mice were given single or in combination treatments of AMD (Amiodarone-HCI injection; Bioniche) and FLC (LKT Laboratories, Inc.) via the intraperitoneal route. AMD treatment doses ranged between 5.0 to 25 mg/kg, while FLC doses ranged from 0.1 to 1 mg/kg in mice infected with FLC-resistant strains and from 5.0 to 40 mg/kg for animals infected with FLC-resistant strains. The treatments were given once daily for 3 days after the first dose. On day 4 postinfection, mice were euthanized, kidneys were harvested and enumerated for C. albicans burdens (41). Combinatorial drug efficacy was assessed by C. albicans kidney burdens reduction in the treated mice relative to the untreated controls.

#### Pseudohyphal and true hyphal growth in liquid media. C. albicans cells were incubated at 37°C in RPMI 1640 (pH 5) for 16 h in the absence or in the presence of different concentrations of AMD (0.5 to 25 μg/mL), FLC (one-half MIC), and AMD-FLC (25 μM and one-half MIC for FLC). All cells were viewed by light microscopy at ×400 to assess pseudohyphal and true hyphal formation.

#### Statistical analysis.

In vitro susceptibility, synergism, and expression profiling experiments were repeated at least three times on different days. Arithmetic means and standard deviations were used to statistically analyze the continuous variables (FIC, expression profiling data, ergosterol percentage, and kidney burdens). Geometric means were used to statistically compare MIC results. In order to approximate a normal distribution, the MICs were transformed to log2 values to establish susceptibility differences between strains. Both on-scale and off-scale results were included in the analysis. The off-scale MICs were converted to the next concentration up or down. The significance of the differences in MICs, FICs, ergosterol content, and kidney burdens was determined by using the Student t test (unpaired, unequal variance). Significant differences in MIC values and in vivo studies were determined by using the Student t test (unpaired, unequal variance) and by the Wilcoxon rank sum test, respectively; a P value of <0.05 was considered significant. Statistical analysis was done with the Statistical Package for the Social Sciences (version 13.0; SPSS, Inc., Chicago, IL).

### Table 1. Antifungal susceptibility testing and drug interaction evaluation of all the strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amino acid substitution in Erg11p</th>
<th>Gene overexpression</th>
<th>MIC</th>
<th>Combination MIC</th>
<th>FLC-AMD interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FLC</td>
<td>AMD</td>
<td>FLC</td>
</tr>
<tr>
<td>36082</td>
<td>None</td>
<td>None</td>
<td>1.00</td>
<td>25.0</td>
<td>0.50</td>
</tr>
<tr>
<td>5314</td>
<td>None</td>
<td>None</td>
<td>0.50</td>
<td>25.0</td>
<td>0.20</td>
</tr>
<tr>
<td>90028</td>
<td>None</td>
<td>None</td>
<td>1.00</td>
<td>25.0</td>
<td>0.50</td>
</tr>
<tr>
<td>1005</td>
<td>None</td>
<td>None</td>
<td>0.50</td>
<td>25.0</td>
<td>0.06</td>
</tr>
<tr>
<td>3795*</td>
<td>F1306/LK134R</td>
<td>None</td>
<td>&gt;128</td>
<td>25.0</td>
<td>0.50</td>
</tr>
<tr>
<td>610†</td>
<td>None</td>
<td>CDR1</td>
<td>0.25</td>
<td>25.0</td>
<td>0.12</td>
</tr>
<tr>
<td>611†</td>
<td>None</td>
<td>CDR1</td>
<td>64.0</td>
<td>25.0</td>
<td>8.00</td>
</tr>
</tbody>
</table>

* MICS and ΣFIC values were obtained for all strains according to CLSI document M27-A3 using RPMI 1640 medium buffered at pH 5.
†, matched clinical isolates (41); ‡, matched clinical isolates (20).
 MICS represent geometric means of at least three MICS determined on different days. Values are expressed in μg/ml for FLC and in μM for AMD. “Combination” refers to the MIC of each drug in combination with the other. These MIC combinations together with the standard MICs were used to obtain the ΣFIC values displayed in the table.
* As reported by F. C. Odds (39).
RESULTS

Antifungal susceptibilities and synergism. The MIC and MFC values for AMD against *C. albicans* showed significant variation as a function of pH (*P* = 0.01) with the lowest values observed at a pH of ≤5 for all strains examined. MFC values were the same as the MIC values demonstrating that AMD has fungicidal properties against *C. albicans*. AMD was ineffective against *C. albicans* at higher pH values (MIC > 800 μM). Conversely, the FLC MICs obtained using RPMI 1640 at pH 7 (CLSI document M27A3 condition) (6) showed no statistically significant differences compared to those obtained at pH 5 (*P* = 0.54). These observations were congruent with previous studies (9, 18, 33, 37). Thus, RPMI 1640 growth medium at pH 5 was adopted for all of the experiments with AMD and FLC, both individually and in AMD-FLC combination. Under these conditions, all strains showed the same AMD MIC (25 μM), whereas the FLC MICs ranged from 0.25 to >128 μg/ml (Table 1). The FLC-susceptible strains tested displayed an AMD-FLC FIC index that was interpreted as synergistic indifference (range, 0.563 to 1.250). On the other hand, AMD-FLC showed a synergistic effect with FLC-resistant strains (FIC ≤ 0.500) (Table 1).

Transcriptional changes underlying AMD-FLC antifungal action against *C. albicans*. Transcriptional profiling was used to assess changes in gene expression associated with the AMD, FLC, and AMD plus FLC treatment in *C. albicans*. Many of the target genes of interest were identified from prior transcriptional microarray studies performed on *S. cerevisiae* (53). Optimal expression profiling growth conditions were established using early time points (5, 10, 15, and 30 min) and different AMD and FLC concentrations (1, 10, 25, and 50 μg/ml) using a control strain ATCC 36082. For AMD, the biggest fold increases in expression were observed at 25 μM (MIC) (Fig. 1A) and when the cells were exposed to AMD for 10 min (Fig. 1B). These data suggest that for several key genes, the transcriptional response reached its peak shortly after drug exposure and diminished over time. For FLC, the biggest differences in expression were seen when strain ATCC 36082 was exposed to 0.5 μg of FLC/ml (one-half MIC). When different time points were evaluated, the highest expression differences were observed at 1 h of FLC exposure (not shown). On the basis of these results, the condition established for analyzing the AMD-FLC combination influence in expression profiling was 1-h FLC (one-half MIC) exposure, followed by 10 min of growth in the presence of 25 μM AMD.

After drug exposure with 25 μM AMD for 10 min, 22 of 59 genes analyzed showed a significant change in expression levels (expression fold change ≥ 2). These changes were bigger for FLC-resistant clinical strains (Table 2). The upregulated genes included genes involved in cell wall organization, Ca²⁺ overload stress response, transcription factors involved in stress response, and calmodulin-calcineurin pathway. Of these categories, the Ca²⁺ homeostasis pathway genes, *RIM101* and *TAC1* stood out. We examined six Ca²⁺ transporter genes, including plasma membrane Ca²⁺ channels (*CCH1* and *MID1*) (15, 29), ATP-driven Ca²⁺ pumps in vacuolar and Golgi membranes (*PMC1* and *PMRI*) (11, 16, 28, 47), a vacuolar Ca²⁺/H⁺ vacuolar exchanger (*VCX1*) (11), and a voltage-dependent, Ca²⁺ selective vacuolar ion conductance channel (*YVC1*) (40). Of these, *CCH1, MID1, PMC1, and PMRI* were upregulated in response to AMD treatment, showing that the AMD mechanism of action is likely linked to Ca²⁺ overload, as observed in *S. cerevisiae* (53). Furthermore, of four genes examined in the calmodulin-calcineurin pathway (*CMD1, CNA1, CNB1, and CRZ1*), *CMD1* and *CRZ1* were strongly activated in response to AMD, supporting the notion that the AMD mechanism of toxicity is mediated by Ca²⁺ overload, as in *S. cerevisiae* (18, 53). AMD altered the expression of other genes involved in cell wall synthesis and regulation, diverse pathway regulation (transcription factors), GTPase activity, pH and cation regulation, vesicle transport, and cycle cell regulation (Table 2). As expected, FLC exposure induced expression of genes for ergosterol biosynthesis pathway (*ERG1, ERG11, ERG3*, and *ERG6*) in the FLC-susceptible strains. On the other hand, *ERG2, ERG7*, and *ERG24* and all of the other categories did not show expression changes after FLC treatment. Unexpectedly, the expression of ergosterol synthesis pathway genes was significantly reduced (*P* < 0.01) by the combination FLC-AMD treatment in the FLC-susceptible strain studies (Table 2). Moreover, all genes overexpressed under AMD treatment showed reduced expression when FLC plus AMD was used (Table 2). In general, this reduction was statistically significant and greater (*P* < 0.01) in the FLC-resistant strains, helping to explain the synergistic *in vitro* effect of FLC-AMD observed in FLC-resistant strains.
AMD and FLC reduce the total ergosterol content in a synergistic manner. All clinical strains and the control strain ATCC 90028 showed 2- to 4-fold more ergosterol than the control strains ATCC 36082 and SC5314 (Table 3). These last two strains showed a massive filamentous form production in the growth condition used, suggesting that ergosterol content is lower in filamentous forms than in planktonic cells. Independent of the FLC susceptibility and filamentous form production, all of the strains showed statistically significant reductions in ergosterol content in the presence of FLC (P < 0.05) (Table 3). As expected, FLC-susceptible strains showed a greater reduction in ergosterol content than FLC-resistant strains after FLC treatment (4- to 1.6-fold and 1.4 to 2.0- fold, respectively). On the other hand, treatment with AMD alone did not affect the ergosterol content, both in FLC-susceptible and -resistant strains, suggesting that AMD does not directly influence the ergosterol pathway. Despite this lack of effect, and in accordance with the \( \Sigma \) FIC values, the ergosterol content decreased more in the FLC-resistant than in FLC-susceptible strains when AMD was used in combination with FLC (Table 3).

### In vivo AMD and FLC synergy studies

A murine model of disseminated candidiasis was used to assess the effect of AMD-FLC combination against FLC-susceptible and -resistant isolates. To observe potential synergy, drug concentrations for AMD and FLC were selected in order to have a minimal effect on kidney burdens. Figure 2 shows that treatment with AMD alone at 25 or 5.0 mg/kg/day for 5 days did not affect kidney burden (P > 0.10). Similarly, FLC at 0.1 mg/kg/day failed to affect kidney burdens. Figure 2 shows that treatment with AMD and FLC synergy studies.
reduce organ burdens when the mice were inoculated with the
FLC-susceptible strain 1002 \( (P = 1.00) \). On the other hand,
when the animals were inoculated with strain 610, the treat-
ment with FLC at 0.1 mg/kg/day produced a 2-fold reduction in
kidney burdens \( (P = 0.008) \). FLC at 1 mg/kg/day showed 2.5-
and 3-log reduction for strains 1002 and 610, respectively.
However, when animals were treated with a combination of
increasing FLC concentrations \( (0.5 \text{ to } 1 \text{ mg/kg/day}) \) in the
presence of fixed amounts of AMD at 5 or 25 mg/kg/day, there
was an FLC dose-dependent reduction in organ burden (Fig.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**FIG. 2.** Efficacy of AMD and FLC combination in a murine candidiasis model. Female BALB/c mice (CRL) were challenged with the
FLC-susceptible *C. albicans* strain 1002 (isogenic with 3795) (A), the FLC-resistant *C. albicans* strain 3795 (with two ERG11 mutations T376C and
A428G) (B), the FLC-susceptible *C. albicans* strain 610 (isogenic with 611) (C), and the FLC-resistant *C. albicans* strain 611 (with overexpression
CDR) (D). At 3 h postinfection, mice were given single or combination treatments of AMD \( (5.0 \text{ to } 25 \text{ mg/kg}) \) and/or FLC \( (0.1 \text{ to } 1 \text{ mg/kg}) \) in mice
infected with FLC-susceptible strains and 5.0 to 40 mg/kg in mice infected with the FLC-resistant strains intraperitoneally. The treatments were
given once daily for 3 days after the first dose. On day 4 postinfection, mice were euthanized, kidneys were harvested and enumerated for *C.
albicans* burdens. White boxes represent kidney burdens in log CFU/g for each mouse; black boxes represent average kidney burden per group.
Tx, treatment.

### TABLE 3. Sterol quantification expressed as a percentage of the cell dry weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Mean % concn ± SD</th>
<th>FLC vs NT</th>
<th>AMD vs NT</th>
<th>FLC-AMD vs NT</th>
<th>FLH-AMD vs FLC</th>
<th>FLH-AMD vs AMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>36082</td>
<td>0.45 ± 0.05</td>
<td>0.11 ± 0.01</td>
<td>0.60 ± 0.01</td>
<td>0.70 ± 0.05</td>
<td>0.0006</td>
<td>0.32</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>0.05</td>
<td>0.00001</td>
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<tr>
<td>I</td>
<td>5314</td>
<td>0.70 ± 0.05</td>
<td>0.40 ± 0.03</td>
<td>0.80 ± 0.01</td>
<td>0.70 ± 0.01</td>
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<td></td>
<td>0.58</td>
<td>0.00001</td>
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<tr>
<td>III</td>
<td>1002</td>
<td>1.25 ± 0.01</td>
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<tr>
<td>III</td>
<td>3795</td>
<td>1.05 ± 0.05</td>
<td>0.75 ± 0.01</td>
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<td>0.005</td>
<td>0.0005</td>
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<tr>
<td>IV</td>
<td>610</td>
<td>1.75 ± 0.05</td>
<td>1.1 ± 0.02</td>
<td>1.45 ± 0.00</td>
<td>0.8 ± 0.02</td>
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<td></td>
<td>0.58</td>
<td>0.0001</td>
</tr>
<tr>
<td>IV</td>
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<td>0.6 ± 0.02</td>
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<td></td>
<td>0.0001</td>
<td>0.58</td>
</tr>
</tbody>
</table>

a NT, no treatment.
b Groups I and II, control laboratory strains; group III, clinical isogenic strains; group IV, clinical isogenic strains; group I, massive filamentous form production; groups II, III, and IV, regular pseudohypha and true hypha production.
2) \((P = 0.008)\). This drug synergy was saturated with AMD at 5 mg/kg/day \((P = 1\) comparing AMD at 25 mg/kg/day and FLC at 1 mg/kg/day to AMD at 5 mg/kg/day and to FLC at 1 mg/kg/day). To explore additional synergy with AMD and FLC in an azole-resistant background, animals were infected with FLC-resistant \(C. albicans\) strains (Table 1). In these strains, due to the strong FLC resistance phenotype, FLC alone had no effect on reducing microbial burdens \((P < 0.05)\). AMD alone at 25 mg/kg/day had no significant affect on microbial burden \((P > 0.05)\). The combination of increasing FLC concentration (5.00 to 40 mg/kg/day) in the presence of AMD at 5 or 25 mg/kg/day showed strong synergy \((P < 0.05)\). When the mice were inoculated with strain 3795, which contains the \(erg11\) mutations \(F136L\) and \(K134R\), the treatment with FLC at 40 mg/kg/day showed a >4-log reduction in kidney burden when combined with AMD at 25 mg/kg/day relative to the no-drug control \((P = 0.016)\). A more pronounced reduction in burden was observed when FLC (40 mg/kg/day) was used in combination with AMD at 5 mg/kg/day \((P = 0.008)\) (Fig. 2). Moreover, in three mice, this combination treatment led to total kidney sterilization. This led to the suggestion that changes in the lanosterol demethylase due to mutation may be important for virulence. When FLC-resistant strain 611, which overexpresses \(CDR1\), was used for infections, the FLC-AMD combination treatment (20 and 5 mg/kg/day) showed a smaller decrease in fungal burden \((P = 0.008)\).

**AMD inhibits filamentous form development in \(C. albicans\).**

The inhibition of filamentous form production was observed in all strains used throughout the present study independent of genotype. Reducing filamentous form production may help account for reduced virulence after drug combination therapy. In all strains, AMD started showing filamentous form inhibition at 1 \(\mu\)M and reached its maximum when AMD was added at a final concentration of 10 \(\mu\)M (Fig. 3). The effect was apparent in multiple strains but was more pronounced in azole-resistant strains (Fig. 3). The molecular mechanism involved in this phenotype could be linked with changes in the expression of four genes related with hyphal and pseudohyphal morphogenesis. The gene \(UME6\), an inductor of hyphal development (52), showed no expression in all of the strains studied in the presence of AMD (Table 2). Moreover, three well-characterized transcription factors, i.e., \(Tup1p\), \(Nrg1p\), and \(Rfg1p\), acting as negative regulators of hyphal and pseudohyphal morphogenesis were upregulated in the presence of AMD (25).

**DISCUSSION**

**AMD mechanism of action in \(Candida albicans\).**

AMD is an antiarrhythmic drug observed to possess antifungal properties against a wide range of fungi \(in vitro\) (9). The mechanism of AMD action has been linked to rapid opening of plasma membrane calcium channels (10, 18, 37), reduced membrane fluid-
ity (2, 46), and disruption of the cell cycle (53) in C. neoformans and S. cerevisiae. Recently, it was reported that the fungicidal effect of AMD in S. cerevisiae is a consequence of calcium stress involving the calcineurin-responsive transcription factor Crz1p (51, 53). The transcriptional profile of key pathway genes in the present study (Table 2) suggests that the antifungal effect of AMD against C. albicans is also most likely mediated by Ca\(^{2+}\) stress, alteration of cell wall organization, disruption of nutrient sensing/signaling, and perturbation of the transcription regulation.

We analyzed six genes encoding Ca\(^{2+}\) pumps, channels, and transporters and a transcription factor (RIM101) involved in the cation overload response (27). This group of genes includes a high-affinity plasma membrane Ca\(^{2+}\) channels (CCH1 and MID1) (15, 29), a vacuolar calcium P-type ATPase essential for Ca\(^{2+}\) homeostasis (PMCl) (16, 28, 47), a Golgi apparatus Ca\(^{2+}\) pump (PMR1) (11, 28), a vacuolar Ca\(^{2+}/H\)\(^{+}\) vacuolar exchanger (VCX1) (11), and a voltage-dependent, Ca\(^{2+}\)-selective vacuolar ion conductance channel (VCV1) (40). CCH1, MID1, PMC1, and RIM101 were overexpressed, strongly suggesting that AMD activity is linked with Ca\(^{2+}\) and pH stress. This proposed mechanism of AMD action in C. albicans was confirmed by the induction of CRZ1 expression in response to this drug. It was demonstrated that when Ca\(^{2+}\) enters the cell using Cch1p channels, the calcineurin signaling pathway is activated and Crz1p is part of this pathway (34).

The implication of Ca\(^{2+}\) in AMD mechanism of action was also evident when the GYP7 gene expression profiling was studied. This gene encodes a putative activator of the vacuolar Ypt17p GTPase inducing an acceleration of its GTPase activity (50). This last protein interacts physically with Ccz1p (for C. albicans Ypt7p GTPase inducing an acceleration of its GTPase activity (27). This group of genes includes RIM101 transcription regulation.

A high-affinity plasma membrane Ca\(^{2+}\) the cation overload response (27). This group of genes includes RIM101 confirmed by the induction of C. albicans This proposed mechanism of AMD action in C. albicans was confirmed by using clonal C. albicans FLC-susceptible and -resistant C. neoformans (1, 17). This effect was confirmed by analyzing the expression of seven genes important for cell wall biosynthesis and the cell integrity signaling pathway (see the supplemental material). TUS1 (a Rho1p exchange factor modulator) and FK52 (a glucan synthase subunit linked with the calcineurin pathway) showed modest but significant up-regulation (Table 2). Hence, AMD may alter cell wall regulation and other differential pathways since Rho1p is a ubiquitous regulatory protein contributing to cell wall integrity, cell polarity, cytoskeleton reorganization, and protein kinase C regulation (13, 14, 31, 45, 48).

**Mechanism of AMD-FLC synergy in C. albicans.** The synergetic effect of the combination AMD and azole drugs was demonstrated in vitro against azole-resistant C. albicans and A. fumigatus (1, 17). This effect was confirmed by using clonal FLC-susceptible and -resistant C. albicans clinical strains. These data show that ERG11 mutations strongly reduce the AMD-FLC FIC values from indifferent to synergistic. However, to examine this further, we explored potential drug synergy in a murine candidiasis model. Drug ranges for both AMD and FLC were selected to have a minimal affect on microbial burdens when used alone. However, in combination, there was a pronounced (≥2-log) reduction in kidney CFU for a wild-type susceptible strain. However, the effects were much more prominent for a FLC-resistant strain, which showed a >4-log reduction in the presence of both drugs (Fig. 2). This apparent discrepancy between in vivo and in vitro data may be explained by the antifilamentous form activity of AMD, which affects virulence. The expression profiling data presented here helps to explain the molecular mechanism of filamentous form inhibition by demonstrating an AMD-dependent upregulation of repressors of genes involved in this cellular response. This fact may increase mouse survival since filamentous form and Ume6p are well-known C. albicans virulence factors (5, 52).

In order to understand the strongest synergistic activity of the FLC-AMD combination in FLC-resistant strains, expression profiling was undertaken using five strains exposed to FLC and AMD alone and in combination. FLC treatment induces the hyperexpression of the ergosterol pathway genes as a compensatory response to reestablish the plasma membrane ergosterol levels. On the other hand, AMD treatment causes the hyperexpression of vacuole Ca\(^{2+}\) pumps and calcineurin pathway genes in order to overcome the Ca\(^{2+}\) overload. These stress responses induced by individual drug treatments were totally or partially inhibited when FLC was used in combination with AMD independently of the FLC susceptibility. However, the combination treatment produced a bigger dampening in the transcriptional response (P < 0.01) in the FLC-resistant strains than in the FLC-susceptible isolates.

The partial or total inhibition of the stress responses induced by the combination treatment shows a possible connection between ergosterol synthesis and Ca\(^{2+}\) homeostasis pathways. This notion was described previously for mammal cells where ketoconazole and other azole derivatives were shown to have a direct effect on the inflammatory response by inhibiting the calcineurin pathway via calcmodulin (19). Moreover, some authors have hypothesized a possible mechanism of triazole-AMD synergism as the AMD inhibition of the pre-lanosterol homeostasis disruptors in the inhibition of S. cerevisiae sterol isomerases (ERG2) (36). However, none of these observations was reproduced in our experiments with C. albicans (Table 2). In this yeast, the synergetic effect of AMD-FLC against FLC-resistant isolates could be explained by an alteration in membrane fluidity and enzyme function produced by the reduction of plasma and organelle membrane ergosterol in the erg11 mutant. A recent report of Maresova et al. (32) suggests that AMD is a membrane active drug that elicits a transient hyperpolarization of the membrane, followed by a depolarization resulting in the influx of Ca\(^{2+}\) and H\(^{+}\) and loss of cell viability. Since ergosterol is the central sterol in fungal cell membranes, it is expected that changes in the ergosterol biosynthetic pathways could have downstream effects on membrane composition resulting in altered membrane fluidity and enzyme function. Ergosterol depletion of organelle mem-
branes impacts vacuolar H\(^+\)-ATPase function, which greatly alter cellular stress responses (Y.-Q. Zhang and R. Rao, unpublished data). Such changes may account for reported synergy observed between AMD and FLC with triazole-resistant strains, especially those with amino acid changes in Erg1p different than Y132H (22, 23, 26). Our ergosterol quantification data suggest that another mechanism may also have to exist to explain the reduction in total ergosterol when AMD-FLC combination treatment was compared to FLC alone against FLC-resistant strains. The somewhat lower but significant synergistic behavior of AMD-FLC treatment against the strain harboring CDR overexpression can be explained by the modulation of efflux pumps induced AMD, as suggested previously for azole-resistant \textit{A. fumigatus} (1).

Overall, these data support a role for AMD and related membrane active agents as a novel adjunct for existing antifungal therapy. The \textit{in vitro, in vivo}, and expression profiling data presented here demonstrate that pathways regulated by AMD could be considered as possible new antifungal targets differing significantly from existing classes of antifungal drugs.

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REFERENCES


