The high mortality rate of mucormycosis with currently available monotherapy has created interest in studying novel strategies for antifungal agents. With the exception of amphotericin B (AMB), the triazoles (posaconazole [PCZ] and itraconazole [ICZ]) are fungistatic in vitro against *Rhizopus oryzae*. We hypothesized that growth at a high temperature (42°C) results in fungicidal activity of PCZ and ICZ that is mediated through apoptosis. *R. oryzae* had high MIC values for PCZ and ICZ (16 to 64 μg/ml) at 25°C; in contrast, the MICs for PCZ and ICZ were significantly lower at 37°C (8 to 16 μg/ml) and 42°C (0.25 to 1 μg/ml). Furthermore, PCZ and ICZ dose-dependent inhibition of germination was more pronounced at 42°C than at 37°C. In addition, intracellular reactive oxygen species (ROS) increased significantly when fungi were exposed to antifungals at 42°C. Characteristic cellular changes of apoptosis in *R. oryzae* were induced by the accumulation of intracellular reactive oxygen species. Cells treated with PCZ or ICZ in combination with hyperthermia (42°C) exhibited characteristic markers of early apoptosis: phosphatidylserine externalization visualized by annexin V staining, membrane depolarization visualized by bis-[1,3-dibutylbarbituric acid] trimethine oxonol (DiBAC) staining, and increased metacaspase activity. Moreover, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay and DAPI (4′,6-diamidino-2-phenylindole) staining demonstrated DNA fragmentation and condensation, respectively. The addition of N-acetylcysteine increased fungal survival, prevented apoptosis, reduced ROS accumulation, and decreased metacaspase activation. We concluded that hyperthermia, either alone or in the presence of PCZ or ICZ, induces apoptosis in *R. oryzae*. Local thermal delivery could be a therapeutically useful adjunct strategy for these refractory infections.

Mucormycosis is a severe opportunistic fungal infection with increasing incidence in immunocompromised hosts (1, 2). *Rhizopus* species are the most common causes of mucormycosis (3). *Mucorales* are resistant to most antifungal agents; only amphotericin B (AMB) and the triazoles posaconazole (PCZ) and itraconazole (ICZ) have in vitro and in vivo activity (4, 5). Thus, the elucidation of novel targets or mechanisms for enhancing the activity of current antifungals could improve outcomes for this devastating infection.

Researchers have shown that hyperthermia induces both necrotic and apoptotic death of malignant cells (6, 7). Apoptosis is a natural homeostatic process in multicellular eukaryotes. Although apoptosis in fungi and mammals has common features, some aspects of the underlying mechanisms of cell death are different, such as caspases (8, 9). Metacaspases are caspase-like cysteine proteases identified in yeasts, plants, and protozoa and distantly related to metazoan caspases (10). Although metacaspases share structural properties with caspases, they lack aspartic acid (Asp) specificity and cleave their targets after Arg or Lys residues. A metacaspase-specific molecular probe for measuring and inhibiting metacaspase activity is not available (10, 11).

Apoptosis has been observed in a number of filamentous fungi, such as *Aspergillus nidulans*, *Aspergillus fumigatus*, *Neurospora crassa*, and *Fusarium oxysporum* (12–15). Various physiological stresses, ionizing radiation, and hyperthermia have been reported to trigger apoptosis in fungi and cancer cells (14–16). However, the precise mechanism of hyperthermia-induced apoptosis in mammalian cells is not clear. Hyperthermia is known to result in expression of heat shock proteins, which regulate the expression of proinflammatory cytokine genes in cancer cells, resulting in apoptosis (7, 16). Moreover, in a mouse model, hyperthermia (42 to 43°C) was shown to induce endotoxin release from the plasmalemma and to increase intracellular Ca^{2+}, inducing apoptosis (7).

In fungi, the calcineurin pathway regulates several processes, such as cation homeostasis, morphogenesis, virulence traits, and antifungal drug resistance (17, 18). Specifically, in pathogenic fungi, the calcineurin pathway functions as an important “circuit” for fungal homeostatic cell responses, which counters the toxic effects of antifungals in the cell membrane and cell wall, thus contributing to antifungal resistance (19–21).

We hypothesized that hyperthermia induces apoptosis in *Rhizopus oryzae* and that this effect is pronounced in the presence of ergosterol biosynthesis inhibitors (PCZ and ICZ). We also tested the hypothesis that the calcineurin inhibitor tacrolimus (TCR), one of the most common immunosuppressants in solid organ and hematopoietic stem cell recipients, acts synergistically with PCZ and ICZ, especially at high temperatures. We found that the combination of hyperthermia and either PCZ or ICZ induced apoptotic events in *R. oryzae* cells, specifically reactive oxygen species (ROS) accumulation, metacaspase activation, and phosphatidyl-
serine externalization, as well as DNA fragmentation and condensation.

**MATERIALS AND METHODS**

**Drugs.** AMB (5 mg/ml; Sigma), fluconazole (FLC) (2 mg/ml; Pfizer), and PCZ (5 mg/ml; Merck & Co., Inc.) were prepared in sterile water. ICZ (5 mg/ml; Janssen Pharmaceuticals) and the calcineurin inhibitor TCR (1 mg/ml; Astellas Pharma Inc.) were prepared in ethanol, and aliquots were stored at −20°C in the dark until use. AMB was used as a positive control, and FLC, which has no activity against *R. oryzae*, was used as a negative control.

**Isolates and growth conditions.** Clinical isolates of *R. oryzae* (R-a-969), *Cunninghamella bertholtiae* (C.b-5633), and *Mucor circinelloides* (M.c-4030) were grown on freshly prepared Sabouraud dextrose agar plates. After 48 h of incubation at 37°C, spores were collected in sterile phosphate-buffered saline (PBS), washed twice in PBS, and counted using a hemocytometer. The spores were stored at 4°C in PBS.

**Susceptibility testing.** Both microbiidilution was performed according to the Clinical and Laboratory Standards Institute method M38-A2 (22). Briefly, 2-fold serial drug (PCZ and ICZ) dilutions in RPMI 1640 medium (1× RPMI buffered medium with 2% glucose) were prepared in flat-bottom 96-well microtiter plates (100 μl/well). Drug-free wells were used as controls. Each well was inoculated with 100 μl of freshly isolated *R. oryzae* spores (3 to 4 days old; 1×107 spores/ml) and incubated in the test medium. After 48 h of incubation at 25°C, 37°C, and 42°C, the MICs of PCZ and ICZ were determined visually as the lowest drug concentrations resulting in 100% growth inhibition. Similarly, the MICs of azoles were determined at 42°C when azoles were used in combination with a synergist concentration of 0.015 μg/ml TCR. To determine the minimum fungicidal concentrations (MFCs) of PCZ and ICZ, an aliquot (20 μl) taken from each well that showed complete inhibition of growth was plated onto YPD agar (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) plates (23). After 24 h of incubation at 25°C, 37°C, and 42°C, the MFC was recorded as the lowest drug concentration at which no growth was observed.

**Germination assay.** To evaluate the effects of antifungals on *R. oryzae* germination, spores (106/ml) were resuspended in drug (PCZ and ICZ)-containing RPMI 1640 medium and incubated at 25°C, 37°C, and 42°C with shaking for 5 h. The medium was removed by centrifugation at 13,000 × g, and the germlings were resuspended in RPMI 1640 medium containing drugs (PCZ and ICZ). Next, the germlings were washed twice in 0.1 M 3-(N-morpholino)propanesulfonic acid (pH 7) (MOPS buffer) and incubated with 2 μg/ml of bis-[1,3-dibutylbarbituric acid]trimethine oxonol (DiBAC) (Molecular Probes), as previously described (25, 26). After 1 h of incubation at room temperature (RT) in the dark, samples were washed twice in MOPS buffer. Images were acquired using a fluorescence microscope (Nikon Microphot SA) with a fluorescein isothiocyanate (FITC) filter.

**Detection of apoptosis markers in *R. oryzae*.** Spores (1×106 spores/ml) were inoculated in RPMI 1640 medium at 25°C, 37°C, and 42°C for 5 h to obtain germlings. The resulting *R. oryzae* germlings were resuspended in RPMI 1640 medium with and without antifungal agents (PCZ and ICZ; 0 to 16 μg/ml) and incubated for 3 h at 25°C, 37°C, and 42°C with shaking (180 rpm). Similarly, germlings were treated with azoles in combination with TCR (0.015 μg/ml) at 42°C. After incubation for 3 h, the germlings were harvested and washed twice with potassium phosphate buffer (pH 7.8; 200 mM) containing sorbitol (PBS; 1 M). The cell wall was digested after incubation with a lysing enzyme mixture (1 U of chitosanase, 1.3 U of chitinase, 1 U of lyticase, and 10 mg/ml lysing enzyme [Sigma]) for 5 h at 30°C (27) and analyzed for the presence of apoptotic markers, as described by Madoe et al. and Shirazi and Kontoyiannis (26, 28, 29).

At the onset of apoptosis in *R. oryzae*, phosphatidylserine was translocated to the plasma membrane surface and detected using annexin V-FITC (Annexin V Apoptosis Detection Kit; BD Pharmingen). Germlings were washed and resuspended in binding buffer (1 mM HEPES-NaOH, pH 7.4, 14 mM NaCl, 0.25 mM CaCl2, in PBS). Five microliters of annexin V-FITC (50 μg/ml) and 5 μl of propidium iodide (PI) (200 μg/ml) were added to 1 ml of cell suspension, and the mixture was incubated for 20 min in the dark at RT (26, 30, 31). The germlings were observed microscopically for fluorescence with excitation and emission settings of 488 nm and 520 nm. The excitation and emission wavelengths for PI were 536 nm and 623 nm, respectively.

DNA fragmentation in *R. oryzae* was detected using a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (26, 31). *R. oryzae* cells were fixed with 3.7% formaldehyde for 30 min on ice and digested with a lysing enzyme mixture. Germlings were rinsed twice with PBS and incubated in a permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice. Next, the cells were rinsed twice with PBS and incubated with 50 μl of DNA-labeling solution for 60 min at 37°C. After incubation, the cells were rinsed three times with PBS and incubated with 100 μl of an antibody solution (anti-bromodeoxyuridine [BrdU]–fluorescein) for 30 min at RT. The cells were then observed microscopically for fluorescence with excitation and emission wavelengths of 488 nm and 520 nm.

To assess chromatin condensation in *R. oryzae* germlings, samples were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma). After being washed with PBS, the cells were incubated with 3 μg/ml DAPI in PBS for 10 min at RT in the dark. The germlings were then observed microscopically for fluorescence with excitation and emission wavelengths of 350 nm and 461 nm.

**Detection of intracellular ROS production.** The amount of ROS in *R. oryzae* germlings was measured using dihydrorhodamine (DHR)-123 (Sigma) staining (26, 31). Germlings treated with PCZ and ICZ alone or in combination with TCR (0.015 μg/ml) at a high temperature (42°C) were spiked with DHR-123 (5 μg/ml) and incubated for 2 h. The germlings were then harvested and viewed directly under a fluorescence microscope equipped with a filter set with an excitation limit of 500 nm and an emission limit of 550 nm.

**Detection of metacaspase activity in *R. oryzae* cells.** Detection of active metacaspases in *R. oryzae* germlings was performed using the CaspASE FITC-VAD-FMK (Promega) according to the manufacturer’s instructions (26, 32). Briefly, germlings treated with PCZ and ICZ at 37°C and 42°C or in combination with TCR at a high temperature (42°C) for 3 h were collected, washed in PBS, resuspended in 10 μM FITC benzyloxy-carbonyl-Val-Ala-Asp (OMe) fluoromethylketone (VAD-FMK), and incubated for 2 h at 30°C. After incubation, the germlings were washed twice in PBS and observed microscopically for fluorescence with excitation and emission settings of 488 nm and 520 nm.

Inhibition of apoptosis was performed by incubating *R. oryzae* germlings with PCZ in the presence or absence of the caspase 1 inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) (Sigma) and the antioxidant N-acetyl-cysteine (NAC) at final concentrations of 40 μM and 40 mM, respectively. After incubation for 3 h, the germlings were spread onto YPD plates and incubated at 37°C and 42°C for 12 h. The cell colonies were counted and expressed in CFU. The numbers of CFU of cells incubated with both PCZ and Z-VAD-FMK or NAC and those incubated with PCZ alone were compared each time.

For all assays, three independent experiments were carried out on 3 different days.

**Statistical analysis.** Comparisons of multiple treatment groups were performed using two-way analysis of variance with Bonferroni’s postest for significance. Calculations were performed using the InStat software program (GraphPad Software). P values of less than 0.05 were considered to be statistically significant.
Enhanced hyphal damage caused by PCZ and ICZ at high temperature correlates with enhanced membrane permeability and increased intracellular ROS accumulation. To further elucidate the mechanism of the fungicidal action of triazoles at 42°C, we stained R. oryzae cells with the membrane potential-sensitive DiBAC, which enters depolarized cells (25). We observed increased DiBAC uptake at 42°C for PCZ or ICZ at all concentrations tested, indicating enhanced hyphal membrane damage and increased cellular permeability compared to an untreated control (8.0% ± 1.0%) (Fig. 1).

As studies have suggested that accumulation of ROS is a key regulator of apoptosis in yeasts (33), we used DHR-123 to assess the ROS accumulation in R. oryzae in the presence or absence of PCZ and ICZ at different temperatures. DHR-123 is oxidized by intracellular ROS and transformed into the fluorescent chromophore Rh-123 (33). R. oryzae cells treated with PCZ and ICZ at 42°C had significantly higher ROS levels than did untreated cells or cells treated with PCZ or ICZ alone and grown at 25°C and 37°C (Fig. 2 and 3 and Table 2; see Fig. S2 in the supplemental material). Specifically, we observed ROS accumulation after treatment with PCZ and ICZ at a concentration of 8 μg/ml at 37°C, whereas a significant increase in fluorescence was detected at much lower drug concentrations (0.25 μg/ml for PCZ and 0.5 μg/ml for ICZ) at 42°C (Fig. 2 and 3 and Table 2). Twenty-two percent to 45% of cells were stained with DHR-123 when exposed to the combination of TCR (0.015 μg/ml) and PCZ or ICZ (0.06 to 0.25 μg/ml) (Table 2). These results demonstrate that accumulation of ROS correlates with PCZ- and ICZ-induced fungicidal activity in R. oryzae at high temperatures. Similarly, concentrations of PCZ and ICZ at much lower MICs had an increased effect on the generation of intracellular ROS in C. bertholletiae and M. circinelloides at 42°C (see Fig. S3 in the supplemental material).

**Evidences of apoptosis induced by PCZ and ICZ in R. oryzae cells at high temperatures.** To confirm the apoptotic features in PCZ- and ICZ-treated R. oryzae cells, we evaluated nuclear DNA fragmentation and condensation using TUNEL assays and DAPI staining, respectively, at different temperatures. PCZ- and ICZ-exposed R. oryzae cells exhibited significant nuclear DNA condensation in a concentration- and incubation temperature-dependent fashion (Fig. 2 and 3 and Table 2). Strong blue fluorescence indicated a greater degree of apoptotic DNA condensation and fragmentation than in the intact nuclei of control cells. Marginal staining of nuclei was observed at 25°C, whereas at 37°C, DNA condensation in the nuclei of 43% to 67% of the R. oryzae cells co-incubated with 8 μg/ml of PCZ or ICZ was observed. At 42°C and low concentrations of PCZ and ICZ (0.25 to 0.50 μg/ml), 54% to 63% of the nuclei were stained with DAPI compared to the untreated control (6% to 18%) (Fig. 2 and 3 and Table 2; see Fig. S2 in the supplemental material).

Similarly, chromatin condensation was observed in 15% ± 1.0% to 35% ± 2.0% of the Mucor and Cunninghamella germlings co-incubated with 4 to 16 μg/ml of PCZ and ICZ at 37°C (see Fig. S3 in the supplemental material). At 42°C and very low drug concentrations (0.125 to 0.50 μg/ml PCZ and ICZ), we observed nuclear staining in 25% ± 2.0% to 40% ± 2.0% of the Mucor and Cunninghamella germlings (see Fig. S3).

We obtained similar results using the TUNEL assay (Fig. 2 and 3 and Table 2). The proportion of TUNEL-positive nuclei was significantly higher (20 to 48%) at 42°C, even in the presence of very low PCZ (0.25 μg/ml) and ICZ (0.5 μg/ml) concentrations.
compared to 25°C, where staining was very faint and the drug concentrations were much higher (Fig. 3 and Table 2; see Fig. S2 in the supplemental material).

As high temperatures induce both apoptosis and necrosis in mammalian cells (15), we sought to differentiate between apoptotic and necrotic *R. oryzae* cells by using the annexin V-FITC and PI double-staining method. In this method, apoptotic cells are stained with annexin V-FITC, whereas PI accumulates in the nuclei of necrotic cells via membrane permeabilization (34). Following PCZ (0.25 \( \mu \)g/ml) and ICZ (0.5 \( \mu \)g/ml) exposure at 42°C, 65% to 76% of the cells were stained with annexin V-FITC, while only 6% to 26% and 19% to 73% of the cells were stained with annexin V-FITC at 25°C (PCZ or ICZ at 8 to 16 \( \mu \)g/ml) and 37°C (PCZ or ICZ at 4 to 8 \( \mu \)g/ml), respectively (Fig. 4 and Table 2). In drug-free

**FIG 1** Altered plasma membrane homeostasis triggered by treatment with AMB, ICZ, and PCZ at 25°C, 37°C, and 42°C. (A to C) Staining with DiBAC showing increased green fluorescence, which indicates loss of viability due to increased membrane permeability. (D to F) Graphs of the percentages of *R. oryzae* germlings displaying staining with DiBAC at different temperatures (25°C, 37°C, and 42°C) compared to untreated control cells at a range of drug concentrations (0 to 16 \( \mu \)g/ml; \( P < 0.048 \)). **, \( P < 0.001 \); ***, \( P < 0.0001 \); NS, \( P > 0.05 \) compared to untreated controls. The experiments were performed in triplicate and repeated three times. The error bars indicate standard deviations (SD). Light microscope images are labeled as DIC (differential interference contrast).

**FIG 2** DNA and nuclear damage and ROS generation in *R. oryzae* cells after treatment with ICZ and PCZ at 37°C visualized using fluorescence microscopy. Shown are TUNEL (A), DAPI (B), DHR-123 (C), and PI (D) staining. The experiments were performed in triplicate and repeated three times.
medium, we did not observe annexin V-FITC staining in cells at 25°C, whereas 3% to 15% of cells were annexin V-FITC and PI positive at 37 and 42°C (Fig. 4 and Table 2). These results suggest that PCZ and ICZ induce apoptosis in *R. oryzae* cells, especially at higher temperatures, which was associated with their fungicidal properties. Similarly, the calcineurin inhibitor TCR (0.015 μg/ml), in combination with PCZ or ICZ (0.06 to 0.25 μg/ml) at a high temperature, induced apoptosis in *R. oryzae* cells (25% to 50%, 25% to 60%, 30% to 45%, and 5% to 25% of cells were stained by TUNEL, DAPI, annexin V, and PI, respectively) (Table 2).

Metacaspases are activated at high temperatures in the presence of PCZ and ICZ. Caspases are activated in the early stages of apoptosis and play a central role in the apoptotic cascade.

**TABLE 2** Effects of PCZ and ICZ on apoptosis of *R. oryzae* at different temperatures

| Temp (°C) | Drug (μg/ml) | % Apoptotic cells
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Annexin</td>
</tr>
<tr>
<td>42</td>
<td>Control</td>
<td>14.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>44.0 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>76.0 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>PCZ + TCRb</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>45.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>30.0 ± 3.0</td>
</tr>
<tr>
<td>37</td>
<td>Control</td>
<td>3.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>19.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>73.0 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>ICZ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>28.0 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>51.0 ± 4.0</td>
</tr>
<tr>
<td>25</td>
<td>Control</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PCZ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>16.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>ICZ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>14.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>26.0 ± 1.5</td>
</tr>
</tbody>
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a ± SD. ND, Not detected.
b TCR, 0.015 μg/ml.
Although caspases are not present in fungi, researchers have identified orthologs of mammalian caspases, called meta-caspases, in fungi and plants, and their activity can be assessed using the *in situ* detection marker CaspACE FITC-VAD-FMK (33). Apoptotic *Candida albicans* cells with activated meta-caspases stained fluorescent green, whereas nonapoptotic cells remained unstained (34). To confirm the presence of meta-caspase activation in *R. oryzae* germlings, we treated them with PCZ (0.25 to 16 µg/ml) and ICZ (0.5 to 16 µg/ml) at 25°C, 37°C, and 42°C and incubated them with CaspACE FITC-VAD-FMK, which binds to active metacaspases (34). No meta-caspase activation was observed at 25°C, whereas concentration-dependent activation was observed at 37°C (20% to 57%) and was more prominent at 42°C (27% to 50%) (Table 2). These results suggest that PCZ and ICZ induce apoptosis via metacaspase activation. TCR, in combination with azoles, further potentiated apoptosis by caspase-like activity in 25% to 45% of *R. oryzae* cells (Table 2).

To further support the concept that caspase-like activities are involved in apoptosis at 42°C, we treated *R. oryzae* germlings with 0.25 µg/ml PCZ for 3 h in the presence or absence of the caspase 1 inhibitor Z-VAD-FMK at a concentration of 40 µM. We assessed the metacaspase activity in Z-VAD-FMK-treated and untreated samples of the *R. oryzae* germlings. At 42°C, cells treated with PCZ in combination with Z-VAD-FMK exhibited higher survival rates, with only 5% apoptotic cells, than did cells treated with only PCZ (50% apoptotic cells). Similarly, the colony formation rate was 78.5% ± 2.0% higher in Z-VAD-FMK-treated cells than in untreated cells. These results further support the notion that PCZ- and ICZ-induced death of *R. oryzae* cells at 42°C requires metacaspase activity (Fig. 6A to C).

Finally, to further elucidate the role of ROS in apoptosis, we examined whether the ROS scavenger NAC reverses apoptosis in *R. oryzae* cells. At 42°C, the levels of intracellular ROS were markedly decreased, apoptosis was inhibited, and the cell survival rate was increased (65% ± 3%), after the addition of the ROS scavenger NAC (40 mM) compared to non-NAC-exposed cells (37% ± 4%) (Fig. 6A, B, and D).

![FIG 4](https://example.com/fig4.jpg) **FIG 4** Representative fluorescence images of ICZ- and PCZ-treated *R. oryzae* cells and untreated control cells at different temperatures (25°C, 37°C, and 42°C) captured using a fluorescence microscope. Annexin V/PI stains show the presence of apoptotic cells (green fluorescence) and necrotic cells (red fluorescence). The experiments were performed in triplicate and repeated three times.

![FIG 5](https://example.com/fig5.jpg) **FIG 5** Effects of treatment with PCZ and ICZ on the activity of metacaspases at 37°C and 42°C in *R. oryzae* germlings as confirmed using FITC-VAD-FMK staining. Activation of metacaspases in *R. oryzae* cells was visualized under a fluorescence microscope. Although control germlings did not exhibit any fluorescence, germlings exposed to PCZ and ICZ exhibited green fluorescence at temperatures of 37°C and 42°C. The experiments were performed in triplicate and repeated three times.
DISCUSSION

Mucormycosis is a severe, frequently lethal infection with a paucity of therapeutic options. Despite aggressive therapy and frequently toxic antifungal agents, the mortality rate from the infection remains high. Therefore, alternative strategies are needed to improve the mediocre activity of our current antifungals against these recalcitrant fungi (35). In the present study, we hypothesized that hyperthermia increases the potency of drugs with otherwise modest activity against Mucorales and that this is mediated via induction of apoptosis. Our findings make induction of apoptosis in fungal cells an attractive pathway for targeting cell death in infection with Mucorales.

We observed for the first time that the growth-inhibitory effect of hyperthermia combined with antifungal drugs resulted in rapid onset of apoptosis in R. oryzae cells. Nakao et al. (16) previously reported that hyperthermia affects tumor cell proliferation and growth. The hyperthermia temperature (42°C) and exposure time (3 h) we used in combination with antifungal drugs were comparable to those used previously with mastocytoma cells, in which apoptosis was induced via heating at 43°C and 44°C for 30 min (16). These findings demonstrate that the optimal conditions for hyperthermia appear to be similar among eukaryotic cells regardless of their origins.

The mechanism of hyperthermia-induced apoptosis in R. oryzae and the fungicidal activity of PCZ and ICZ were correlated with increased ROS accumulation. Furthermore, exogenous supplementation of the ROS scavenger NAC not only reduced ROS accumulation, caspase activation, and apoptosis, but also increased survival. Studies have linked ROS formation, cytochrome c release, and changes in mitochondrial membrane potential with yeast apoptosis (34, 36). Furthermore, authors have reported that cells, when heated to temperatures of 41°C to 47°C, expressed markers of apoptosis, while temperatures higher than these led to necrosis (37). It was also reported that noxious stimuli, including heat and cold, caused proapoptotic proteins to induce caspase 2, 8, or 9 expression and that this expression eventually led to apoptosis via mitochondrial damage (37). Furthermore, opening of pores in the mitochondrial membrane led to activation of proapoptotic factors, which decreased the cells’ membrane potential (37).

Fungi have a class of caspase-related proteases called metacaspases (10). In the present study, pretreatment of R. oryzae cells with the broad-spectrum caspase inhibitor Z-VAD-FMK and the ROS scavenger NAC blocked ROS accumulation, indicating that PCZ or ICZ, along with hyperthermia, inhibited the growth of R. oryzae and triggered apoptosis via activation of a caspase-like enzyme.

Experimental evidence presented here points to the central role of ROS as an inducer of apoptosis under hyperthermic conditions with or without the use of triazoles. Authors have also reported that ROS-induced apoptosis occurs in A. nidulans, F. oxysporum, and C. albicans cells (13, 15, 38). Intracellular accumulation of ROS is one of the major stimuli for induction of apoptosis in eukaryotes (9). Sharon et al. (15) reported that apoptotic pathways in fungi seem to be mitochondrion dependent. Mitochondrial respiration is considered to be a powerful source of superoxide radicals in miconazole- and farnesol-induced apoptosis (39, 40). Additionally, hyperthermia is reported to participate in the intrinsic mitochondrial pathway by the generation of ROS rather than in the extrinsic death receptor pathway in nucleated cells, supporting our findings (41).

Calcineurin is known to regulate pathogenesis and morphogenesis in a temperature-dependent fashion in C. albicans, Cryptococcus neoformans, and Paracoccidioides brasiliensis (17–21). We found that the calcineurin inhibitor tacrolimus had a profound

FIG 6 Effects of PCZ-treated R. oryzae cells on apoptosis in the presence of Z-VAD-FMK and NAC. PCZ-treated R. oryzae germlings with and without Z-VAD-FMK and NAC at 37°C and 42°C were collected, stained with FITC-VAD-FMK and DHR-123, and observed under a fluorescence microscope. (A, a, B, and b) Control germlings. (C and c) Germlings exposed to 8 and 0.25 μg/ml PCZ and 40 μM Z-VAD-FMK. (D and d) Cells exposed to 8 and 0.25 μg/ml PCZ and 40 mM NAC at 37°C and 42°C, respectively. The experiments were performed in triplicate and repeated three times.
fungidal and proapoptotic activity against *Mucorales* at a high temperature compared to azoles alone. These data suggest that the calcineurin pathway plays a key role in the regulation of *R. oryzae* susceptibility to azoles at high temperatures.

Experimental applications of hyperthermia alone or in combination with drugs or nanoparticles in cancer treatment are emerging (42, 43). The literature also contains reports of the potential value of hyperthermic therapy for infectious diseases by capitalizing on the difference in thermotolerance between normal and infected cells (43). Thus, our findings support the idea that local thermal delivery of triazoles and other antifungal drugs deserves further study, as it may be a useful adjunct therapeutic strategy for refractory *Mucorales* infections. Although the data presented here are an important proof of concept, *in vivo* studies are needed to confirm the effectiveness of hyperthermia as an adjunct therapeutic modality that could be applicable in clinical practice.

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