The calcineurin pathway regulates antifungal drug resistance and the virulence of several major human-pathogenic fungi, including the recalcitrant Mucorales. We hypothesized that the fungistatic triazoles posaconazole (PCZ) and itraconazole (ICZ) become fungicidal in the setting of the calcineurin inhibitor tacrolimus (TCR) and that such an effect is mediated through apoptosis. Fungicidal activity and apoptosis were studied using standard microbiological techniques and hypal metabolic and vital dye reduction assays at 37°C in RPMI 1640. Apoptosis was characterized by detecting intracellular Ca^{2+}, phosphatidylserine (PS) externalization, DNA fragmentation, plasma membrane integrity, chromatin condensation, reactive oxygen species (ROS) generation, caspase-like activity, ATP, and cytochrome c release. MICs for PCZ and ICZ alone were significantly higher (8 to 128 μg/ml) than those of PCZ or ICZ plus TCR (0.25 to 4 μg/ml) for Rhizopus oryzae, Cunninghamella bertholletiae, and Mucor circinelloides. Both PCZ and ICZ in combination with TCR became fungicidal, and their activity was mediated through increased apoptotic cell death of R. oryzae (10 to 50%), C. bertholletiae (5 to 50%), and M. circinelloides (5 to 55%) germlings, with morphological apoptotic changes characterized by externalization of PS, nuclear condensation, and DNA fragmentation. Moreover, activation of the caspase-like activity was correlated with cell death induced by TCR plus PCZ or ICZ. These changes correlated with elevated intracellular Ca^{2+} and ROS levels and disturbance of mitochondrial potential. We found that PCZ or ICZ in combination with TCR renders Mucorales sensitive to triazoles via apoptotic death. These observations could serve as a new paradigm for the development of new therapeutic strategies.
MATERIALS AND METHODS

Drugs. PCZ (5 mg/ml; Merck & Co., Inc.) and fluconazole (FLC; 2 mg/ml; Pfizer) stocks were prepared in distilled water. ICZ (5 mg/ml; Janssen Pharmaceuticals) and calcineurin inhibitor TCR (1 mg/ml; Astellas Pharma Inc.) stocks were prepared in ethanol, and aliquots of these were stored at −20°C in the dark until use.

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

Susceptibility testing. broth microdilution was performed according to the Clinical and Laboratory Standards Institute method (20). Briefly, 2-fold serial drug (PCZ, ICZ, and FLC) dilutions were prepared in flat-bottomed 96-well microtiter plates (100 µl/well) in the presence or absence of a synergic concentration of TCR (0.015 µg/ml). Drug-free wells were used as controls. Each well was inoculated with 100 µl of freshly isolated *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* spores (2 to 3 days old, 1 × 10^4 spores/ml) suspended in RPMI 1640. After 48 h of incubation at 37°C, the MICs of PCZ, ICZ, and FLC were determined visually as the lowest drug concentrations resulting in complete growth inhibition. To determine the minimum fungicidal concentrations (MFCs) of PCZ, ICZ, and TCR, an aliquot (20 µl) taken from each well that showed 100% growth inhibition was plated onto YPD agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) plates. After 24 h of incubation at 37°C, the MFC was recorded as the lowest drug concentration at which no growth was observed.

Viability assay. *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings treated with TCR along with PCZ (0.125 to 4 µg/ml) or ICZ (0.06 to 4 µg/ml) for 3 h were stained with bis-(1,3-dihydroxy-2-naphthalenyl)disulfonic acid (DiBAC4; Molecular Probes) as previously described (21). PCZ and ICZ concentrations were decided on the basis of their MIC values.

Ca²⁺ detection in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides*. By adding exogenous CaCl₂, Lu et al. (22) showed that intracellular Ca²⁺ levels and apoptosis are increased and that depletion of Ca²⁺ levels by EDTA leads to decreased apoptosis in *Candida albicans*. We assessed the effect of TCR on Ca²⁺ levels and apoptosis in the presence of PCZ or ICZ. Briefly, germlings were loaded with Ca²⁺-detecting stain 5 µM Fluo-3/AM (Molecular Probes) and incubated for 30 min at 37°C to detect Ca²⁺ levels by fluorescence microscopy. The fluorescence intensity of Ca²⁺-stained *C. bertholletiae* was measured using fluorescence microscopy.

Annexin V–PI double staining of *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* cells. The apoptosis marker phosphatidylserine (PS) is located on the inner leaflet of the lipid bilayer of the cytoplasmic membrane and is translocated to the outer leaflet at the onset of apoptosis (16–18). Cells treated with PCZ (0.06 to 0.5 µg/ml; C. bertholletiae, 1 to 4 µg/ml; M. circinelloides, 1 to 4 µg/ml) or ICZ (0.06 to 0.25 µg/ml; C. bertholletiae, 0.25 to 1 µg/ml; M. circinelloides, 1 to 4 µg/ml) in combination with TCR (0.015 µg/ml) were digested with a lysis enzyme mixture (1 U of chitosanase, 1.3 U of chitinase, 1 U of lyticase, 10 mg/ml lysis enzyme; Sigma) for 5 h at 30°C. After digestion, cells were stained with annexin V–fluorescein isothiocyanate (FITC; BD Pharmingen) and propidium iodide (PI) at room temperature (RT) for 15 min and observed under a fluorescence microscope to assay the externalization of the apoptosis marker PS, as previously described (17, 19).

Detection of intracellular ROS accumulation and ΔΨm in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings. Reactive oxygen species (ROS) play an important role as an early initiator of apoptosis in yeast and other filamentous fungi, including the *Mucorales* (19, 23). We used the mitochondrial membrane potential (ΔΨm), an indicator for the energetic state of the mitochondria and the depolarization of the mitochondrial membrane (24). Intracellular ROS levels and ΔΨm in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings were measured after treatment with PCZ (0.125 to 4 µg/ml) or ICZ (0.06 to 4 µg/ml) in combination with TCR (0.015 µg/ml) for 3 h at 37°C using a fluorometric assay with dihydrodorhodamine-123 (DHR-123) and rhodamine-123 (Rh-123) (Sigma) staining (19, 25).

Measurement of DNA damage in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides*. DNA fragmentation, a characteristic change in apoptosis, was detected using a terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* (17, 19, 26). Cells pretreated with PCZ (0.06 to 0.5 µg/ml; C. bertholletiae, 1 to 4 µg/ml; M. circinelloides, 1 to 4 µg/ml) or ICZ (0.06 to 0.25 µg/ml; C. bertholletiae, 0.25 to 1 µg/ml; M. circinelloides, 1 to 4 µg/ml) in conjunction with TCR (0.015 µg/ml) for 3 h at 37°C were fixed with 3.7% formaldehyde for 30 min on ice and were digested with the lysing enzyme mixture. Lysed cells were used to detect DNA fragmentation by the TUNEL assay as described by Madoe et al. (17). The cells were observed for fluorescence with excitation and emission wavelengths of 488 nm and 520 nm, respectively.

Chromatin condensation and fragmentation are well-described cytoplasmic hallmarks of apoptosis (17). Chromatin condensation was assessed in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings pretreated with PCZ (0.125 to 4 µg/ml) or ICZ (0.06 to 4 µg/ml) in combination with TCR (0.015 µg/ml) for 3 h at 37°C. After incubation, germlings were stained with 3 mg/l of 4′,6-diamino-2-phenylinodole (DAPI; Sigma) in PBS for 10 min at RT in the dark (16–19). Germlings were then observed for fluorescence with excitation and emission wavelengths of 350 nm and 461 nm, respectively.

Detection of metacaspase (caspase-like) activity in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings. Metacaspases are caspase-like cysteine proteases identified in yeasts, plants, and protozoa (27). These enzymes are closely associated with the generation of ROS and mitochondrial dysfunction (26). Caspase-like activity was measured in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings pretreated with PCZ (0.125 to 4 µg/ml) or ICZ (0.06 to 4 µg/ml) in conjunction with TCR (0.015 µg/ml) for 3 h at 37°C using the CaspACE FITC-benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (VAD-FMK) in situ marker (Promega, Madison, WI), according to the manufacturer’s instructions (28).

cyt c release from mitochondria. Translocation of mitochondrial cytochrome c (cyt c) to the cytosol is considered a critical event in apoptosis (26). For estimation of cyt c, isolation of mitochondria from *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings was performed according to the method described by Niimi et al. (29). We used *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings grown in RPMI 1640 broth at 37°C for 5 h and then resuspended in fresh RPMI 1640 broth. The germlings were further incubated with PCZ (0.06 to 0.5 µg/ml; C. bertholletiae, 1 to 4 µg/ml; M. circinelloides, 1 to 4 µg/ml) or ICZ (0.06 to 0.25 µg/ml; C. bertholletiae, 0.25 to 1 µg/ml; M. circinelloides, 1 to 4 µg/ml) in the presence of TCR (0.015 µg/ml) for 3 h at 37°C. The quantities of cyt c in the supernatant and mitochondria were determined by the method of Niimi et al. (29). The absorbance at 550 nm was measured with a POLARstar Omega spectrophotometer.

Measurement of ATP release in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings. We measured intracellular ATP efflux from germlings exposed to PCZ (0.06 to 0.5 µg/ml; C. bertholletiae, 1 to 4 µg/ml; M. circinelloides, 1 to 4 µg/ml) or ICZ (0.06 to 0.25 µg/ml; C. bertholletiae, 0.25 to 1 µg/ml; M. circinelloides, 1 to 4 µg/ml) in combination with TCR (0.015 µg/ml) at 37°C for 3 h as an indication of cell membrane damage and cytoplasmic and mitochondrial membrane leakage (21). *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings were separated from the medium by centrifugation and resuspended in 1-ml aliquots of RPMI 1640. The cells were removed by centrifugation, and the supernatants were assayed for ATP using a CellTiter-Glo luminescent kit (Promega, Madison, WI). Data were collected with a microplate luminometer (Spectramax M5; Molecular Devices, Sunnyvale, CA).

Quantification of protein. Protein was estimated according to a method described by Lowry et al. (30), using crystalline bovine serum albumin as a standard.
RESULTS

Azoles (PCZ, ICZ), when combined with TCR, have a profound fungicidal activity against *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* growth. Compared to PCZ alone, PCZ-TCR demonstrated 16-, 2-, and 2-fold higher potency against clinical isolates of *R. oryzae*, *C. bertholletiae*, and *M. circinelloides*, respectively (Table 1). The MFCs of ICZ-TCR against clinical isolates of *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* were 64-, 32-, and 32-fold higher than those of ICZ alone, respectively. TCR had no fungicidal activity over the range of concentrations tested.

DiBAC vital staining also revealed enhanced uptake in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings exposed to PCZ or ICZ in combination with TCR compared to that for the germlings exposed to TCR only (Fig. 1). The results showed an increase in plasma membrane depolarization, as evidenced by an increase in the fluorescence intensity of germlings in comparison to that of germlings treated with TCR (Fig. 1). Similar effects were observed with PCZ or ICZ alone at a higher concentration (8 μg/ml) (data not shown).

### TABLE 1

*In vitro* antimicrobial activity of PCZ and ICZ in combination with TCR against *R. oryzae*, *C. bertholletiae*, and *M. circinelloides*

| Drug(s) | *R. oryzae* MIC (μg/ml) | *C. bertholletiae* MIC (μg/ml) | *M. circinelloides* MIC (μg/ml) |
|---------|-------------------------|-------------------------------|-------------------------------|
| PCZ     | 8.0 (32)                | 8.0 (32)                      | 8.0 (64)                      |
| PCZ-TCR | 0.5 (2)                 | 4.0 (16)                      | 4.0 (16)                      |
| ICZ     | 16.0 (32)               | 32.0 (64)                     | 128.0 (>128)                  |
| ICZ-TCR | 0.25 (1)                | 1.0 (8)                       | 4.0 (16)                      |

*a* Fluconazole had no effect on any test organism under all conditions tested.

*b* MFCs (in μg/ml) are given in parentheses.

*c* TCR was used at 0.015 μg/ml.

Statistical analysis. For all assays, three independent experiments were carried out on 3 different days. Means were compared by two-way analysis of variance with Dunnett’s test for *post hoc* pairwise comparisons. Calculations were performed using the InStat software program (GraphPad Software). *P* values of less than 0.05 were considered to be statistically significant.

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**FIG 1** Fungicidal action of PCZ or ICZ in combination with TCR against *R. oryzae* (A), *C. bertholletiae* (B), and *M. circinelloides* (C) germlings. (A to C) Increased fluorescence of *Mucorales* treated with the triazole-TCR combination compared to that of TCR alone, as shown by the moribidity stain DiBAC. DIC, differential interference contrast. (D to F) Relative fluorescence of *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings, respectively, stained with DiBAC. The experiments were performed in triplicate and repeated three times. *P* values are in comparison to the results for the TCR-treated controls: *, *P* < 0.05; **, *P* < 0.001; ***, *P* < 0.0001; NS, not significant (*P* > 0.05). Error bars indicate standard deviations.
Efflux of ATP in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings treated with PCZ or ICZ in combination with TCR. The rapid efflux of ATP was detected after the incubation of *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings with PCZ or ICZ in combination with TCR. The ATP concentration in the culture supernatant peaked after 3 h of incubation with PCZ (5- to 40-fold) or ICZ (10- to 130-fold) in combination with TCR (Fig. 2A to C). The increased extracellular ATP levels indicate an increase in plasma membrane permeability and decreased intracellular ATP levels of germlings incubated in the presence of PCZ or ICZ with TCR. Our results suggest significant plasma and mitochondrial membrane damage, leading to induction of cell death.

PCZ or ICZ in combination with TCR increases cyt c release from mitochondria in *Mucorales* germlings. We found that mitochondrial cyt c levels were decreased while cytosolic cyt c levels were increased in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings treated with PCZ or ICZ in conjunction with TCR compared to the levels in germlings treated with TCR alone. Specifically, the relative percentage of cyt c in *Mucorales* mitochondria decreased by 1-fold (PCZ) to 2-fold (ICZ) when PCZ and ICZ were used in combination with TCR compared to that with treatment TCR alone. However, cytosolic cyt c levels were higher than mitochondrial cyt c levels, indicating that treatment with PCZ or ICZ in combination with TCR induced the release of cyt c from mitochondria in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings (Fig. 2D to F). The translocation of cyt c from mitochondria to the cytoplasm, which is a pivotal event in apoptosis, leads to ROS production and metacaspase activation.

Azoles (PCZ, ICZ), when combined with TCR, increase intracellular Ca\(^{2+}\) levels in *R. oryzae*, *C. bertholletiae*, and *M. circinelloides* germlings. In *Saccharomyces cerevisiae*, elevation of intracellular Ca\(^{2+}\) leads to cell death, and the increase in intracellular Ca\(^{2+}\) limits cell viability.

**FIG 2** PCZ or ICZ in combination with TCR results in increased cyt c release from mitochondria and ATP release from *R. oryzae* (A, D), *C. bertholletiae* (B, E), and *M. circinelloides* (C, F) germlings. P values are in comparison to the results for the TCR-treated controls: ***, P < 0.001; ****, P < 0.0001; NS, not significant (P > 0.05). Error bars indicate standard deviations. RLU, relative light units.
ular Ca\(^{2+}\) levels indicates that free Ca\(^{2+}\) acts as an initiator of apoptosis (11). We determined intracellular Ca\(^{2+}\) levels upon PCZ or ICZ treatment in combination with TCR using the fluorescent calcium indicator Fluo-3/AM. In the presence of TCR alone, the intracellular levels of Ca\(^{2+}\) in \textit{R. oryzae}, \textit{C. bertholletiae}, and \textit{M. circinelloides} germlings were low and nearly undetectable. After treatment with PCZ or ICZ in combination with TCR for 3 h, all groups showed elevation of intracellular Ca\(^{2+}\) levels (Fig. 3A to F).

PCZ or ICZ in combination with TCR induces morphological characteristics of apoptosis in \textit{R. oryzae}, \textit{C. bertholletiae}, and \textit{M. circinelloides} germlings. Phosphatidylserine (PS) externalization at the outer leaflet of the cytoplasmic membrane is considered an early marker of apoptosis in eukaryotic cells. In fungi, the exposure of PS is typically detected by annexin V-FITC staining. In this assay, the apoptotic cells were detected using staining with annexin V-FITC (green fluorescence), whereas necrotic cells accumulate only PI (red fluorescence). In the plasma membrane, the distribution of phosphatidylserine between the two leaflets is regulated by three enzymes: a Ca\(^{2+}\)-dependent scramblase (a protein responsible for the translocation of phospholipids between the two monolayers of a lipid bilayer of a cell membrane), the ATP-dependent aminophospholipid translocase, and flippase (31). Scramblase activity is dependent on Ca\(^{2+}\), whereas aminophospholipid translocase activity is inhibited by Ca\(^{2+}\) (31). In \textit{R. oryzae}, \textit{C. bertholletiae}, and \textit{M. circinelloides}, elevation of the cytosolic Ca\(^{2+}\) levels with PCZ or ICZ in combination with TCR triggered large amounts of PS exposure (10 to 55%) compared to those for TCR-treated germlings. Therefore, it is possible that PS exposure in apoptosis is Ca\(^{2+}\) dependent (Fig. 4A to F), although the exact mechanism by which Ca\(^{2+}\) regulates PS exposure in Mucorales remains to be elucidated.

Azoles (PCZ, ICZ) in combination with TCR increase intracellular ROS accumulation and mitochondrial potential \((\Delta \Psi_{m})\) in \textit{R. oryzae}, \textit{C. bertholletiae}, and \textit{M. circinelloides} germlings. ROS play important roles as early initiators of apoptosis in yeasts and other filamentous fungi (26). Intracellular ROS levels in \textit{R. oryzae} germlings were measured using a fluorimetric assay with DHR-123 (Sigma) staining (17). An increase of intracellular ROS levels (increase of red fluorescence intensity) was observed in \textit{R. oryzae}, \textit{C. bertholletiae}, and \textit{M. circinelloides} germlings upon PCZ or ICZ exposure in combination with TCR (see Fig. S1 in the...
supplemental material). Compared to the TCR-treated control, at a concentration of 0.125 to 2.0 \( \mu g/ml \) of PCZ or ICZ in combination with TCR, the proportion of fluorescent germlings increased from 5 to 35%. Likewise, \( R. \) oryzae germlings treated with PCZ and ICZ alone at much higher concentrations (8 \( \mu g/ml \)) had higher ROS levels than untreated controls (data not shown). These results indicate that azoles in combination with TCR induce ROS accumulation, which is related to apoptosis.

As shown in Fig. S2 in the supplemental material, PCZ or ICZ in combination with TCR caused a decrease in mitochondrial potential in a dose-dependent manner, as evidenced by an increase in the number of fluorescent germlings in comparison to that for the controls treated with TCR only. After Rh-123 staining, the mitochondrial membrane depolarization was demonstrated by an increase of green fluorescence intensity. The mean fluorescence of \( R. \) oryzae, \( C. \) bertholletiae, and \( M. \) circinelloides germlings treated with PCZ and ICZ in combination with TCR was increased by 2-fold (see Fig. S2 in the supplemental material) compared to that for germlings treated with PCZ or ICZ alone (1- to 1.5-fold) at much higher concentrations (8 \( \mu g/ml \)). These results suggest that PCZ and ICZ trigger depolarization of mitochondria, activating the apoptotic signaling machinery by inducing cation overload or anion efflux. Apoptotic cell death induced by TCR with PCZ or ICZ was attenuated by the presence of cycloheximide (50 \( \mu g/ml \)) (data not shown), indicating that active protein synthesis is required for the induction of apoptosis by TCR with PCZ or ICZ.

**Azoles (PCZ, ICZ) in combination with TCR induce morphological changes of apoptosis in \( R. \) oryzae, \( C. \) bertholletiae, and \( M. \) circinelloides.** Our results with mitochondrial damage, ROS accumulation, and \( Ca^{2+} \) intracellular release prompted us to examine if DNA fragmentation occurs under those conditions. DNA strand breaks were detected by labeling free 3\(^{-}\)OH termini with FITC-labeled deoxyuridine, catalyzed by terminal deoxynucleotidyltransferase, to give green fluorescence. DNA condensation was visualized by labeling cells with the DNA-binding fluorescent dye DAPI. In the untreated cells, chromatin appeared as single round nuclei, whereas a more intensive fluorescence indicated a typical apoptotic DNA condensation in the nuclei of \( M. \) corales exposed to PCZ or ICZ with TCR.

DNA and nuclear fragmentation was assessed in \( R. \) oryzae, \( C. \) bertholletiae, and \( M. \) circinelloides germlings treated with PCZ or ICZ in combination with TCR with the TUNEL assay and the DNA-binding fluorescent dye DAPI. We found that \( R. \) oryzae, \( C. \) bertholletiae, and \( M. \) circinelloides cells treated with PCZ or ICZ in combination with TCR had a TUNEL-positive phenotype in 10\% \pm 1.0\% to 50\% \pm 3.0\% of the cells and chromatin condensation in 10\% \pm 1.0\% to 45\% \pm 5.0\% of the cells (Fig. 5A to F; see Fig. S3 in the supplemental material), indicating DNA fragment-
tation and margination. In control germlings treated with TCR only, chromatin appeared as a single round spot with a normal appearance. In contrast, germlings exposed to PCZ or ICZ plus TCR showed chromatin fragments dispersed in the cells (see Fig. S3 in the supplemental material). Similarly, chromatin condensation was observed in 40% to 60% of the cells coincubated with 8 to 16 g/ml PCZ and ICZ alone at 37°C (data not shown). The condensation and fragmentation of nuclear DNA are the cytological hallmarks of apoptosis. These cytological markers of apoptosis showed that exposure of \textit{R. oryzae}, \textit{C. bertholletiae}, and \textit{M. circinelloides} cells to PCZ or ICZ with TCR resulted in apoptotic DNA damage, an important phenomenon of late-stage apoptosis.

\textbf{Azoles (PCZ, ICZ) in combination with TCR activate caspase-like activity in} \textit{R. oryzae}, \textit{C. bertholletiae}, and \textit{M. circinelloides} \textbf{germlings}. Caspases are activated at the early stages of apoptosis, play a central role in the apoptotic cascade, and can be detected by FITC-VAD-FMK, an FITC conjugate of the caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) that binds specifically to activated caspases (26). Cells with activated intracellular metacaspases have green fluorescence, whereas control cells appear unstained. To look for the presence of caspase-like activity in \textit{R. oryzae}, \textit{C. bertholletiae}, and \textit{M. circinelloides} germlings, we treated germlings with PCZ and ICZ in combination with TCR and incubated them with CaspACE FITC-VAD-FMK for 2 h at 30°C (Fig. 6A to F). A concentration-dependent increase in caspase activation was observed in PCZ- and ICZ-treated \textit{R. oryzae} germlings (1.4- to 1.97-fold), \textit{C. bertholletiae} (1.2- to 1.8-fold), and \textit{M. circinelloides} (1.2- to 1.95-fold) (Fig. 6A to C). No caspase-like activity was detected in controls treated with TCR only. These results indicate that azoles in combination with TCR induce metacaspase activation.

\textbf{DISCUSSION}

We hypothesized that TCR enhances the potency of ergosterol biosynthesis inhibitors PCZ and ICZ, to the point that they become fungicidal, and this activity is mediated through apoptosis in Mucorales. The calcineurin pathway has been shown to be important for survival of pathogenic fungi, because of its central role in various cell processes, including morphogenetic transition and the development of antifungal tolerance and resistance (32–34). Calcineurin pathway inhibition in combination with classical antifungal agents could have broad therapeutic potential in fungal infections (7, 33). Due to the immunosuppressive properties of calcineurin inhibitors, a useful clinical use of a TCR-triazole com-
Combination for mucormycosis would ultimately require a novel agent that selectively targets fungal calcineurin pathways without having collateral effects on human cells.

We found evidence of PCZ-TCR and ICZ-TCR synergism in vitro in several Mucorales species (R. oryzae, C. bertholletiae, and M. circinelloides) tested, in agreement with the findings for other fungal species (12, 13). In addition, we used multiple markers of apoptosis to implicate apoptosis as a mechanism of PCZ-TCR- or ICZ-TCR-induced cell death. The rate of early apoptosis was corroborated by assays for detection of PS by annexin V-FITC, ROS production (DHR-123 staining), and mitochondrial membrane potential, and the rate of late apoptosis was corroborated by assays for DNA damage, nuclear fragmentation (TUNEL and DAPI staining), cytochrome c and ATP release, and Ca^{2+} levels. In each of the assays, apoptosis was evident at PCZ-TCR or ICZ-TCR concentrations that were below the MICs for triazoles (0.25 to 2 μg/ml). Taken together, the data suggest that apoptosis correlated with increased membrane disruption, increased intracellular Ca^{2+} levels, externalization of PS, DNA fragmentation, and accumulation of ROS (Table 1; Fig. 1 to 5; see Fig. S1 and S2 in the supplemental material). Lu et al. (22) showed that increased apoptosis correlated with an increase in intracellular Ca^{2+} levels in C. albicans. Similarly, our results indicate that apoptotic death could be induced in the Mucorales through increasing the intracellular Ca^{2+} level by a combination of drugs (PCZ or ICZ with TCR). Translocation of mitochondrial cyt c to the cytosol leads to its binding with apoptotic protease-activating factor to form a complex with caspase-9, resulting in activation of the caspase cascade (26, 28). Release of cyt c requires an increase in mitochondrial membrane permeability during apoptosis (28). Our results show that, as in mammalian and yeast cells, ROS formation, changes in ΔΨm, and cyt c release also correlate with apoptosis in Mucorales (35, 36). Authors have also reported ROS-induced apoptosis in Aspergillus nidulans, Fusarium oxysporum, and Candida albicans cells (37–39). Sharon et al. (40) reported that apoptotic pathways in fungi seem to be mitochondrial dependent, and mitochondria are considered to be a powerful source of superoxide radicals in miconazole- and farnesol-induced apoptosis (41).
There is accumulating evidence in the baker’s yeast and other eukaryotes that different stimuli induce different apoptotic pathways (42, 43). In mammals, apoptosis is mediated by activation of caspases, which cleave specific substrates and trigger cell death. Caspases are classes of cysteine-aspartic acid proteases regulated at the posttranslational level. When they are cleaved they convey a signal in a proteolytic cascade that induces apoptosis and leads to cell death (44). In the past few years, it has become evident that caspase-like proteolytic activity might exist not only in multicellular organisms but also in unicellular organisms, such as fungi. In this study, we found that, upon treatment with PCZ or ICZ plus M. circinelloides germwals were much higher than those in the TCR-only-treated control. Further studies are needed to demonstrate how proteases contribute to apoptotic fungal death.

In conclusion, we show for the first time that the coadministration of inhibitors of the calcineurin and ergosterol biosynthesis pathways induces apoptosis in Mucorales. This fungicidal synergistic interaction deserves further study, as it may be a useful adjunct therapeutic strategy (15) for mucormycosis.

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