Rapamycin Exerts Antifungal Activity In Vitro and In Vivo against Mucor circinelloides via FKBP12-Dependent Inhibition of Tor

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Departments of Molecular Genetics and Microbiology,a Medicine,a and Pharmacology and Cancer Biology,b Duke University Medical Center, Durham, North Carolina, USA

The zygomycete Mucor circinelloides is an opportunistic fungal pathogen that commonly infects patients with malignancies, diabetes mellitus, and solid organ transplants. Despite the widespread use of antifungal therapy in the management of zygomycosis, the incidence of infections continues to rise among immunocompromised individuals. In this study, we established that the target and mechanism of antifungal action of the immunosuppressant rapamycin in M. circinelloides are mediated via conserved complexes with FKBP12 and a Tor homolog. We found that spontaneous mutations that disrupted conserved residues in FKBP12 conferred rapamycin and FK506 resistance. Disruption of the FKBP12-encoding gene, fkbA, also conferred rapamycin and FK506 resistance. Expression of M. circinelloides FKBP12 (McFKBP12) complemented a Saccharomyces cerevisiae mutant strain lacking FKBP12 to restore rapamycin sensitivity. Expression of the McTor FKBP12-rapamycin binding (FRB) domain conferred rapamycin resistance in S. cerevisiae, and McFKBP12 interacted in a rapamycin-dependent fashion with the McTor FRB domain in a yeast two-hybrid assay, validating McFKBP12 and McTor as conserved targets of rapamycin. We showed that in vitro, rapamycin exhibited potent growth inhibitory activity against M. circinelloides. In a Galleria mellonella model of systemic mucormycosis, rapamycin improved survival by 50%, suggesting that rapamycin and nonimmunosuppressive analogs have the potential to be developed as novel antifungal therapies for treatment of patients with mucormycosis.

Comparative genomics to study closely related organisms has emerged as an important tool for understanding phenotypic differences, such as pathogenicity, and allows for identification of conserved molecular pathways that can be targeted in the development of broad-spectrum antimicrobial drugs. Conserved protein kinases controlling growth and proliferation of fungal pathogens represent attractive drug targets, both because they are likely to be essential for fungal propagation and development and because these enzymes are likely to be conserved among fungal species, allowing the use of inhibitors of these kinases as broad-spectrum antimicrobial therapeutic agents.

Among the repertoire of conserved protein kinases essential for cellular physiology, the Tor protein kinase is the focus of intense investigation, given its role as a central element of nutrient-regulated cell growth pathways. In Saccharomyces cerevisiae, Tor inhibition by the natural product rapamycin elicits many of the cellular responses that are triggered by nutrient starvation, such as inhibition of protein synthesis, downregulation of amino acid permeases, protein degradation, autophagy, and cell cycle arrest (reviewed in reference 45). These effects are mediated largely by the activity of Tor in promoting the expression of genes encoding tRNAs, ribosomal proteins, rRNAs, and amino acid permeases and suppressing nitrogen catabolite repression and the amino acid general control response (4, 6, 10, 22, 29, 41). In S. cerevisiae, two Tor proteins, Tor1 and Tor2, form two distinct multiprotein complexes, named TORC1 and TORC2 (33, 57). Collectively, these complexes control protein synthesis, mRNA synthesis and degradation, ribosome biogenesis, nutrient transport, and autophagy (TORC1), as well as actin polarization and cell wall integrity (TORC2) (reviewed in reference 60).

The Tor kinase has also received wide attention as an antifungal target due to its inhibition by the natural product rapamycin. Rapamycin was first identified as an antimicrobial with potent activity against Candida albicans (1, 56). Subsequently, rapamycin was shown to have robust antifungal activity against several human fungal pathogens, such as Cryptococcus neoformans, Aspergillus fumigatus, Fusarium oxysporum, and several pathogenic Penicillium species (13, 58), and it was later found to have potent immunosuppressive activity (35). In yeast and mammalian cells, rapamycin inhibits Tor through its association with the prolyl isomerase FKBP12, forming a binary complex that binds to the highly conserved FRB (FKBP12-rapamycin binding) domain of Tor. This mechanism of action is supported by the identification of mutations in the FRB domain that confer rapamycin resistance by blocking FKBP12-rapamycin binding to Tor and by structural studies defining the molecular details of FKBP12-rapamycin inhibition of Tor (5, 11, 23, 34, 52, 59).

FKBP12 catalyzes cis-trans peptidyl-prolyl isomerization, a rate-limiting step in protein folding (reviewed in reference 27). Remarkably, FKBP12 also serves as the receptor for the antimicrobial and immunosuppressive drug FK506. Both rapamycin and FK506 bind to the active site of FKBP12 and inhibit its prolyl isomerase activity. The target of FKBP12-FK506 is calcineurin, a Ca2+-calmodulin-regulated serine-threonine-specific protein phosphatase consisting of a catalytic A (CnA) and a regulatory B (CnB) subunit (32). In humans, calcineurin regulates nuclear localization of the transcription factor NFAT during the response to antigen presentation (32). In S. cerevisiae, calcineurin regulates cation homeostasis and cell integrity via the transcription factor Crz1 (46). In C. neoformans, mutants lacking calcineurin are via-

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ble at 24°C but inviable at 37°C, and they are avirulent in animal models of cryptococcosis (18, 39).

While the mechanism of rapamycin action has been conserved in both ascomycete and basidiomycete fungal species (3, 13, 15), little is known about its activity in basal fungal lineages, such as the zygomycetes. One group within the zygomycetes is the order Mucorales, which arose early during fungal radiation and has little is known about its activity in basal fungal lineages, such as the

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phycomyces blakesleeanus strain NRRL1555 (−)</td>
<td>Wild type</td>
<td>Santiago Torres-Martinez</td>
</tr>
<tr>
<td>Rhizopus oryzae strain FGSC9543</td>
<td>Wild type</td>
<td>Santiago Torres-Martinez</td>
</tr>
<tr>
<td>Mucor circinelloides f. lusitanicus strainsb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R7B (−)</td>
<td>leuA− (−)</td>
<td>Santiago Torres-Martinez</td>
</tr>
<tr>
<td>NRRL3631 (+)</td>
<td>Wild type (+)</td>
<td>Santiago Torres-Martinez</td>
</tr>
<tr>
<td>SM2</td>
<td>NRRL3631 (+): fkbA-1(A316G)</td>
<td>This study</td>
</tr>
<tr>
<td>SM4</td>
<td>R7B (−): fkbA-2(L91P)</td>
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</tr>
<tr>
<td>MU402</td>
<td>R7B (−): pyrG−</td>
<td>36</td>
</tr>
<tr>
<td>RMB1</td>
<td>MU402: fkbA::pyrG</td>
<td>This study</td>
</tr>
<tr>
<td>RMB2</td>
<td>MU402: fkbA::pyrG</td>
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<tr>
<td>Saccharomyces cerevisiae strains</td>
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<tr>
<td>JK9-3da</td>
<td>MATα his4 HMLα leu2-3,112 rme1 trp1 ura3-52</td>
<td>23</td>
</tr>
<tr>
<td>JHY3-3B</td>
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<tr>
<td>CHY251</td>
<td>MATα his3Δ200 leu2-3,112 trp1Δ101 ura3-52 pyr6Δ::TRP1</td>
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<tr>
<td>CHY516</td>
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<td>SMY4-1</td>
<td>MATα trp1-901 his3 leu2-3,112 ura3-52 ade2 gal4 gal80 URA3::GAL-lacZ</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>LYS2::GAL-H33 TOR1-3 fpr1::ADE2</td>
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</tbody>
</table>

The indicated strains were kindly provided by Santiago Torres-Martinez, Departamento de Genética y Microbiología, Facultad de Biología, Universidad de Murcia, Murcia, Spain. Mating type for Mucor strains is indicated by “−” or “+”.

We found that rapamycin’s antifungal action is mediated via FKBP12-rapamycin inhibition of Tor, based on the isolation and characterization of spontaneous FKB12 mutations that confer resistance to rapamycin. We also demonstrate that M. circinelloides Tor (McTor) and FKB12 interact in the presence of rapamycin and that disruption of the FKB12-encoding gene, fkbA, confers rapamycin and FK506 resistance. Finally, we show that rapamycin treatment improves survival of the invertebrate Galleria mellonella in a model of systemic mucormycosis, suggesting a therapeutic effect of rapamycin. In summary, this study demonstrates the high conservation of rapamycin action among fungal pathogens, providing insights for novel therapeutic vantage points.

**MATERIALS AND METHODS**

**Strains, media, drugs, and growth conditions**. Strains used in this study are listed in Table 1. Strains were grown at 30°C or room temperature on YPD (1% yeast extract, 2% Bacto peptone, and 2% dextrose), potato dextrose agar (PDA), and RPMI (RPMI 1640 liquid medium with glutamine and without sodium bicarbonate [Gibco], supplemented with 2% dextrose and buffered with 0.165 M MOPS [morpholinepropanesulfonic acid] (pH 7)) media. Strains were grown either on solid media containing 2% agar or in liquid cultures. Rapamycin (LC Laboratories) was dissolved in 90% ethanol-10% Tween 20. FK506 (Prograph) was obtained from the Duke University Medical Center pharmacy. Rapamycin and FK506 stock solutions were added to media at the indicated concentrations.

**Antifungal drug testing.** Drug interactions were assessed by a checkerboard titration following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) (11a) for antifungal susceptibility testing of molds. In vitro testing was performed in RPMI 1640 medium. Aliquots of 50 μl of each drug at a 4× concentration were dispensed to wells of a 96-well microtiter plate to provide 77 drug combinations. Additional rows were used to determine the MIC of each agent alone and for the growth control well (drug-free). Final drug concentrations tested were as follows: rapamycin, 200 μg/ml to 3.12 μg/ml (6 dilutions); and FK506,
25.0 µg/ml to 0.1 µg/ml (9 dilutions). To each well, an inoculum of 5 × 10⁴ sporangiospores/ml was added, and microtiter plates were incubated at 35°C without shaking for 24 h. Growth inhibition determinations were performed with the aid of a concave mirror. The MIC and MIC₉₀ of drugs were defined as the lowest drug concentrations in the wells producing a complete absence of visual growth and an 80% reduction of growth, respectively.

**Identification of M. circinelloides FKBp and Tor homologs.** M. circinelloides FK506 binding proteins were identified by searching the Mucor circinelloides CBS277.49 genome database (http://genome.jgi-psf.org/Mucc2/Mucc2.home.html) for genes encoding proteins containing predicted FK506 binding domains with high homology to the FK506 binding domain of S. cerevisiae FKBP12 (ScFKBP12; encoded by FPR1) (see Fig. 2). Percent identity and similarity were determined by BLAST protein sequence alignments utilizing b2seq from NCBI. Protein domains were identified using the Prosite database of protein domains, families, and functional sites (http://prosite.expasy.org/). The Tor homolog was identified by searching the Mucor circinelloides CBS277.49 genome database for proteins containing a Tor FRB domain with high homology to the S. cerevisiae Tor FRB domain. Phylogenetic reconstructions were performed with the Geneious (Biomatters) platform for sequence alignment, utilizing the Blossom 62 cost matrix and the Jukes-Cantor method for genetic distance modeling.

**Plasmid construction.** The fkbA open reading frame was PCR amplified from an R7B cDNA library by use of primers containing a hemagglutinin (HA) epitope sequence (directly upstream of the fkbA coding sequence) and PstI and SalI restriction sites. The amplicon was digested with PstI and SalI and cloned into PstI/SalI-digested pCu415-HA-FKBP12 (Stratagene, La Jolla, CA). The FRB domain encoding region of torA was amplified from R7B genomic DNA by use of primers containing a Myc epitope sequence (directly upstream of the FRB domain sequence) and EcoRI and PstI restriction sites. The amplicon was first digested with EcoRI, blunted with T4 DNA polymerase, and digested with PstI. The digested amplicon was cloned into Smal/PstI-digested pCu414-MyC-FRB. The yeast two-hybrid construct pGBK7-TFRB was generated by first amplifying the FRB domain sequence of torA from R7B genomic DNA by use of primers containing Ncol and BamHI restriction sites. The amplicon was then digested with Ncol and BamHI and ligated in frame with the GAL4 DNA binding domain (BD) into Ncol/BamHI-digested pGBK7 (Clontech Laboratories Inc.). The pGADT7-FKBP2 plasmid was generated by amplifying the fkbA coding sequence from pCu415-HA-FKBP12 by use of primers containing 30 bp of pGADT7 AD sequence (Clontech Laboratories Inc.) directly upstream of the EcoRI site (including the EcoRI site) and 30 bp of pGADT7 AD sequence directly downstream of the BamHI site (including the BamHI site). The amplicon was cotransformed with EcoRI-digested pGADT7 AD into S. cerevisiae strain SMY4-1 (Table 1) by a standard lithium acetate transformation method. Strains in which the fkbA coding sequence was cloned in frame with the GAL4 activation domain (AD) in pGADT7 AD by homologous recombination were selected on complete synthetic medium lacking leucine (CSM—Leu). The presence of the cloned fkbA coding sequence was confirmed by colony PCR. Total genomic DNA was extracted from positive transformants, and 2 µl of this DNA was transformed into Escherichia coli DH5α cells (Invitrogen Corporation) to recover pGADT7-FKBP2 plasmids.

**Yeast two-hybrid assay.** The two-hybrid strain SMY4-1 (Table 1) was cotransformed with the two-hybrid fusion plasmids pGBK7-TFRB and pGADT7-FKBP12, cells were grown on CSM—Leu—Trp, and β-galactosidase activity was assayed using the chlorophenol-β-D-galactopyranoside (CPRG) substrate as previously described (8).

**Molecular analysis of spontaneously drug-resistant M. circinelloides isolates.** Spontaneously FK506 and rapamycin cross-resistant strains were isolated by spreading 500 M. circinelloides sporangiospores on YPD solid medium containing 1 µg/ml FK506 and passing FK506-resistant mycelial outgrowths on FK506-containing solid medium until homokaryotic mycelial growth was observed. Spore suspensions were collected and tested for rapamycin resistance on YPD solid medium containing 100 µg/ml rapamycin. Total DNA was isolated from M. circinelloides mycelia germinated in liquid YPD medium at 30°C by harvesting mycelia over Miracloth (Calbiochem, La Jolla, CA) and vacuum drying mycelia by lyophilization. Dried mycelia were triturated with glass beads by manual agitation and resuspended in 1 ml CTAB extraction buffer (100 mM Tris-HCl [pH 8.4], 1.4 M NaCl, 25 mM EDTA, 2% cetyltrimethylammonium bromide [CTAB]). A standard phenol-chloroform extraction procedure was used, followed by DNA precipitation using a 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. The DNA pellet was resuspended in 1 ml of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0). Genomic DNA was used to amplify 700-bp fragments by PCR, including 1-kb sequences upstream and downstream of the fkbA coding sequence, and each fragment was sequenced. DNA analysis was compared by genomic DNA BLAST searches against the M. circinelloides genomic database sequences.

**Gene expression analyses.** M. circinelloides cultures were grown in liquid YPD medium at 30°C, and mycelium powder extracts were prepared as described above. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions, and 30 µg of total RNA was loaded onto a 1% formaldehyde agarose gel. Following transfer, membranes were hybridized to radioactive fkbA and actA DNA-based probes. Hybridized probe signals were detected using a phosphorimager. cDNA was synthesized from 1.5 µg of total RNA by use of an AffinityScript multiple-temperature reverse transcriptase kit (Stratagene).

**Western blot analyses.** M. circinelloides protein extracts were generated from cultures grown in YPD medium at 30°C by harvesting mycelia over Miracloth (Calbiochem, La Jolla, CA) and vacuum drying mycelia by lyophilization. Dried mycelia were triturated with glass beads by manual agitation, and 20 mg was resuspended in 200 µl of sample buffer (1% SDS, 9 M urea, 25 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.7 M beta-mercaptoethanol) supplemented with phenylmethylsulfonyl fluoride (PMSF) and the indicated concentration of Roche Complete EDTA-free protease inhibitor cocktail. Samples were boiled for 2 min, vortexed for 1 min, and boiled again for 1 min. An additional centrifugation step was carried out for 15 min at 16,000 rpm, and supernatants were collected. For Western blotting, 0.3 mg of total protein extract was loaded on a 4 to 20% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ). Blots were blocked with 5% nonfat milk-0.05% Tween 20 in Tris-buffered saline, and membranes were incubated overnight at 4°C with a 1:1,000 dilution of antiserum against ScFKBP12, raised in rabbits (7). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody was used as the secondary antibody, at a dilution of 1:2,000. Equal loading of samples was monitored by stripping and incubating membranes for 1 h with a 1:2,000 dilution of a rat anti-alpha-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). HRP-conjugated anti-rat IgG antibody was used as the secondary antibody, at a 1:2,000 dilution. Proteins were visualized by using ECL Western blotting substrate (Pierce Biotechnology, Rockford, IL) following the manufacturer’s protocol.

**S. cerevisiae total protein extracts were prepared by disrupting cells with glass beads in lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 100 µM Na₃VO₄, 25 mM beta-glycerophosphate, 25 mM NaF) supplemented with PMSF and the indicated concentration of Roche Complete EDTA-free protease inhibitor cocktail. Fifty micrograms of total protein extract was used for Western blotting as described above.**

**Disruption of the M. circinelloides fkbA gene.** An R7B fkbA disruption cassette was generated by overlap PCR amplification of an amplicon containing 1,000 bp of fkbA genomic DNA sequence directly upstream of the ATG codon, with an added XbaI restriction site; a 2,000-bp pyrG gene amplicon; and an amplicon with 1,000 bp of fkbA genomic DNA sequence directly downstream of the fkbA coding sequence stop codon, with an added Xmal restriction site. The resulting fkbA::pyrG disruption cassette
Formation was performed as described in reference 55, with a modified germinated spores were incubated with 5 mg/ml chitosanase (molecular grade; U.S. Biologicals RD) and 0.5 mg/ml lysing enzymes (Sigma) for 90 min at 30°C. The disruption plasmid was linearized with XhoI and dephosphorylated with calf intestinal phosphatase (CIP; New England BioLabs), and 50 μg of digested plasmid was transformed into two independent MU402 (R7B pycG− strain) (Table 1) cultures. Transformation was performed as described in reference 55, with a modified protoplast preparation step as described in reference 37, in which 2.5 × 10^8 germinated spores were incubated with 5 mg/ml chitosanase (molecular grade; U.S. Biologicals RD) and 0.5 mg/ml lysing enzymes (Sigma) for 90 min at 30°C. Rba disruption was verified by PCR (see Fig. 6B) utilizing primers P1 (JHHE20967; 5′-GAAATAAGAAAGATAAATGATTAC-3′), P2 (JHHE20968; 5′-AAGCCCTTTATTTGAGATAGAAC-3′), P3 (JHHE2096; 5′-AAAGTATATACAAAGAACATATGACATT-3′), and P4 (JHHE20966; 5′-ATTAGGACATAATTTCATTTGTTAGAATGAC-3′) and by Southern blot analysis (data not shown).

**Galleria mellonella model of systemic zygomycosis.** M. circinelloides inoculums were prepared in phosphate-buffered saline (PBS) by suspending sporangiospores harvested from mycelial lawns grown on PDA for 5 days at room temperature, and spore titers were determined by hemocytometer counting. Drug stocks were prepared by dilution in 90% ethanol-10% Tween 20. Measurements of the hemolymph volume of 20 larvae of various weights and determination of the mean hemolymph volume/kg of body weight by linear regression analysis were used to determine the drug doses administered. Larvae in the final instar were obtained from Vanderholt, Inc. Ten larvae (300 mg ± 25 mg) were used per group. Each larva was injected with 5 μl of PBS containing 500 sporangiospores, PBS only, drugs, or vehicle into the hemocoel via a proleg. At 4 h postinfection, 5 μl of PBS containing drugs or vehicle control was injected into each larva via a separate proleg from the one used during infection. Larvae were incubated at room temperature, and the number of dead larvae was scored daily. Kill curves were plotted, and differences in survival rates (log rank test) were determined by the Kaplan-Meier method, using GraphPad Prism software.

**RESULTS**

**Growth of representative zygomycete species is sensitive to rapamycin.** While the mechanisms of action of rapamycin (sirolimus) and FK506 (tacrolimus) have been established for several ascomycetous and basidiomycetous fungal pathogens, little is known about the activities of these antifungal agents and their mechanisms of action in basal fungal lineages, which include several fungal pathogens of clinical and environmental importance.

To further assess the activities of these natural products among basal fungal species, growth assays of the zygomycete species *M. circinelloides*, *Rhizopus oryzae*, and *Phycomyces blakesleeanus* were performed in the presence of both rapamycin and FK506. Growth of *M. circinelloides* strains R7B (− mating type) and NRRL3631 (+ mating type) and of *R. oryzae* and *P. blakesleeanus* strains was evaluated on rich agar media containing rapamycin or FK506. While all four strains exhibited reduced radial mycelial growth in the presence of rapamycin, *P. blakesleeanus* growth exhibited the highest sensitivity to rapamycin, with a clear reduction in both radial mycelial growth and apical sporangiophore formation (Fig. 1 and Table 2). As observed with rapamycin, FK506 strongly inhibited the growth of all zygomycete species tested (Fig. 1 and Table 2).

In addition, we also determined the rapamycin and FK506 MICs for inhibition of growth of all three zygomycete species, following CLSI’s guidelines for antifungal susceptibility testing of molds by broth microdilution (11a). We found that rapamycin inhibited up to 80% of growth at concentrations above 6.26 μg/ml, 12.5 μg/ml, and 100 μg/ml for *P. blakesleeanus*, *R. oryzae*, and *M. circinelloides*, respectively (Table 3). As expected, *P. blakesleeanus* growth inhibition required lower concentrations of rapamycin, while surprisingly, *M. circinelloides* growth inhibition required high concentrations of rapamycin during liquid culture growth (Table 3). Interestingly, growth of *R. oryzae* exhibited the least sensitivity to FK506 on agar medium containing FK506 (Fig. 2).

**TABLE 2** Radial colony diameters of zygomycete species grown in the presence of YPD, rapamycin, and FK506

<table>
<thead>
<tr>
<th>Strain</th>
<th>YPD</th>
<th>YPD + rapamycin</th>
<th>YPD + FK506</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mucor circinelloides</em></td>
<td>7.2 (0.077)</td>
<td>4.9 (0.080)</td>
<td>0.6 (0.042)</td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td>8 (0)</td>
<td>3.7 (0.086)</td>
<td>0.5 (0.030)</td>
</tr>
<tr>
<td><em>Phycomyces blakesleeanus</em></td>
<td>8 (0)</td>
<td>4.0 (0.061)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Spores (500 spores per plate) from representative zygomycete species were germinated on YPD agar containing either 100 nM rapamycin or 1 μg/ml FK506. Spores were germinated for 72 h at room temperature, and colony diameters were measured.

TABLE 3 Rapamycin and FK506 MIC<sub>90</sub> during growth of zygomycete species

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mucor circinelloides</em></td>
<td>100–200</td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td>12.5–25</td>
</tr>
<tr>
<td><em>Phycomyces blakesleeanus</em></td>
<td>6.3–12.5</td>
</tr>
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* Drug dilutions tested ranged from 200 to 0.09 μg/ml.
1), while it exhibited a higher sensitivity to FK506 than those of the _Mucor_ and _P. blakesleeanus_ strains in a broth microdilution assay (Table 3). While the source of these discrepancies remains unknown, they are likely a result of differences in _R. oryzae_ growth properties on semisolid surfaces and liquid medium. Nevertheless, both assays clearly indicate that _R. oryzae_ is highly susceptible to FK506. In contrast to the fungicidal activity of rapamycin against the pathogens _C. albicans_ and _C. neoformans_ (MICs of \(0.09 \mu g/ml\) and \(0.19 \mu g/ml\), respectively [15]), zygomycete growth was not entirely inhibited by rapamycin, and inhibition required significantly higher concentrations of drug, which could be due partly to decreased permeability of zygomycete cell walls and/or membranes to rapamycin or higher-affinity drug pumps or to occlusion of the FRB domain in zygomycete Tor homologs. Nevertheless, the effect of rapamycin on the growth of the tested zygomycete species provides evidence that in these organisms, Tor also functions to control growth. The MIC\(_{50}\) of FK506 were significantly lower than those observed for rapamycin (Table 3), in agreement with the phenotypes observed during growth on FK506-containing agar medium. These results illustrate that FK506 exhibits potent antifungal activity against the zygomycete species tested.

The _M. circinelloides_ FKB12 homolog and the FRB domain of Tor are required for rapamycin action. We found that growth of _M. circinelloides_ strains was compromised during rapamycin treatment. The presumed targets of rapamycin in this fungal species are the FKB12 and Tor homologs. Comparative analysis of the _M. circinelloides_ genome identified a family of 5 genes encoding 5 putative proteins with closely related FK506 binding domains. We named these genes _fkbA_, _fkbB_, _fkbC_, _fkdD_, and _fkdE_ (FK506 binding), and based on the predicted molecular weights of the proteins they encode, we named their products _FKBP12_, _FKBP19_, _FKBP22_, _FKBP30_, and _FKBP42_, respectively (Fig. 2). FK506 binding proteins with various molecular weights are ubiquitous in nature, and members of the FKBP family have been identified in multiple organisms, including humans, plants, and fungi (40). _FKBP12_ family members contain only one FK506 binding domain, while FKBPs with high molecular weights possess extra domains, such as tetratricopeptide repeat domains and calmodulin binding and transmembrane motifs (20). Based on the 12-kDa predicted molecular mass of the _M. circinelloides_ putative homolog and on phylogenetic analyses of the _Mucor_ _FKBP_ family of proteins and several fungal _FKBP12_ homologs (Fig. 3), we identified _fkbA_ as the gene encoding the _Mucor_ _FKBP12_ homolog.

To test whether the product of _fkbA_ functions as the rapamycin/FK506 receptor in _M. circinelloides_, we performed functional complementation studies using _S. cerevisiae_ as a heterologous host. From an _M. circinelloides_ (R7B) cDNA library, we amplified an HA-tagged _fkbA_ coding sequence amplicon and cloned it into a yeast low-copy-number plasmid under the control of an inducible copper promoter (pCu415CUP1) (30). The resulting plasmid (pCu415-HA-FKBP12) was introduced into a rapamycin-resistant _S. cerevisiae_ strain lacking the FKB12 homolog (_fpr1Δ_23) and tested on rapamycin-containing YPD agar plates with or

<table>
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<th>FKB family member</th>
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<th>Identity</th>
<th>Similarity</th>
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<td>S. c. FKB12 (12 kDa)</td>
<td>26 114</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>M. c. FKB12 (12 kDa)</td>
<td>20 108</td>
<td>75%</td>
<td>83%</td>
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<td>M. c. FKB19 (19 kDa)</td>
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<td>34%</td>
<td>57%</td>
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<tr>
<td>M. c. FKB22 (22 kDa)</td>
<td>47 146</td>
<td>54%</td>
<td>72%</td>
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<td>M. c. FKB30 (30 kDa)</td>
<td>33 122</td>
<td>41%</td>
<td>59%</td>
</tr>
<tr>
<td>M. c. FKB42 (42 kDa)</td>
<td>285 370</td>
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**FIG 2** _M. circinelloides_ family of FK506 binding proteins. Family members were identified based on their protein sequence homology to the FK506 binding domain of _S. cerevisiae_ FKB12. Identity and similarity scores correspond to similarities between the protein sequence of the FK506 binding domain of each _M. circinelloides_ FKB family member and the corresponding domain of _S. cerevisiae_ FKB12. The nomenclature for each FKB (FK506 binding protein) family member is based on its predicted molecular mass (denoted in a parenthesis below each homolog). Protein IDs are from the _Mucor circinelloides_ CBS277.49 v 2.0 genome database, hosted at the Joint Genome Institute (JGI) website. TPR, tetratricopeptide repeat.
without exogenous copper. Heterologous expression of McfkA restored rapamycin sensitivity in the fpr1Δ strain, indicating that McHA-FKBP12 can functionally complement an S. cerevisiae strain lacking endogenous FKBP12 (Fig. 4A).

We also tested whether McHA-FKBP12 can mediate FK506-dependent inhibition of S. cerevisiae calcineurin by transforming the McfkA-containing construct into an S. cerevisiae vph6Δ fpr1Δ double mutant strain (25). Loss of VPH6 (which encodes a component of the vacuolar H\(^+\)-ATPase assembly complex) results in a synthetic lethal phenotype during inhibition of calcineurin by FK506, whereas a vph6Δ fpr1Δ mutant strain also lacking FKBP12 is FK506 resistant (see Fig. S1A in the supplemental material) (24). Surprisingly, heterologous expression of McHA-FKBP12 did not render the vph6Δ fpr1Δ strain sensitive to FK506 (see Fig. S1), indicating that McHA-FKBP12 is unable to inhibit calcineurin function during FK506 exposure, even though the McHA-FKBP12 protein was stably expressed in a vph6Δ fpr1Δ background in a Western blot (data not shown). This may reflect differences in the composite FKBP12-FK506-calcineurin binding surface that arose during divergence of S. cerevisiae and M. circinelloides from their last common ancestor.

Genome analysis also revealed the presence of one gene encoding a Tor homolog (Mucor circinelloides CBS277.49 protein ID 152074), which we designated torA. The FRB domain of Tor was PCR amplified from M. circinelloides (R7B) genomic DNA and cloned into a yeast low-copy-number plasmid containing a copper-inducible promoter (pCu414CUP1) (30). A wild-type S. cerevisiae strain (JK9-3da) was transformed with this plasmid and tested on rapamycin-containing YPD agar in the presence and absence of exogenous copper. In the presence of copper, expression of the M. circinelloides FRB domain rescued the wild-type strain from the fungicidal activity of rapamycin, while a wild-type strain transformed with vector alone remained sensitive to rapamycin (Fig. 4B). These results indicate that the M. circinelloides FRB domain can effectively compete for rapamycin binding (when overexpressed in the presence of copper) and is therefore the likely target of the FKBP12-rapamycin complex in M. circinelloides.

The yeast two-hybrid reporter system was used to determine whether McFKBP12 interacts with the FRB domain of McTor in the presence of rapamycin. To this end, a rapamycin-resistant yeast two-hybrid host strain containing a TOR1-3 mutation and lacking endogenous FKBP12 was employed (SMY4-1) (8). This strain was transformed with plasmids expressing McFKBP12 fused to the GAL4 AD (pGADT7-FKBP12) and the McTor FRB domain fused to the GAL4 BD (pGBK7-FRB), and interactions were quantified by monitoring expression of a GAL4-lacZ reporter gene. Strong interactions between FKBP12 and the FRB domain were observed only in the presence of rapamycin, indicating that the FRB domain of Tor is a target for rapamycin when Tor is complexed with FKBP12 (Fig. 4C). In summary, the McFKBP12 homolog can function as the receptor for rapamycin and can mediate inhibition of Tor via the FRB domain in a heterologous host.

**Mutations in fkbA confer rapamycin and FK506 resistance.** The observation that McFKBP12 can mediate rapamycin inhibition of ScTor yet is unable to promote inhibition of S. cerevisiae calcineurin in the presence of rapamycin prompted us to employ a genetic approach to assess whether McFKBP12 functions as the rapamycin/FK506 receptor in M. circinelloides. Exploiting the robust antifungal activity of FK506 toward M. circinelloides (Fig. 1 and Tables 2 and 3), we isolated a series of strains in two independent strain backgrounds, R7B (− mating type) and NRRL3631 (+ mating type), that exhibited spontaneous FK506 resistance, and we examined the fkbA locus for the presence of genetic lesions.

![FIG 3 Phyllogenetic reconstruction of M. circinelloides FKBP family of proteins and fungal FKBP12 homologos. The McFKBP12 homolog was identified by protein sequence comparisons with *Phycomyces* blakeleanus (P. b.), *Candida* glabrata (C. g.), *S. cerevisiae* (S. c.), *Schizosaccharomyces* pombe (S. p.), *Aspergillus* fumigatus (A. f.), *Neurospora* crassa (N. c.), *Ustilago* maydis (U. m.), *Cryptococcus* neoformans (C. n.), *Rhizopus* oryzae (R. o.), and *Batrachochytrium* dendrobatidis (B. d.) FKBP12 homologs.](http://ec.asm.org/)
Two isolates were identified: one, SM2 (NRRL3631 background), was cross resistant to rapamycin and FK506, and a second isolate, SM4 (R7B background), was sensitive to rapamycin and resistant to FK506 (Fig. 5A). Both isolates were sensitive to the calcineurin inhibitor CsA, indicating that these are not multidrug-resistant isolates (Fig. 5A). SM2 contains an \( fkbA \) allele (\( fkbA-1 \)) harboring an A-to-G substitution (A316G) in the acceptor splice site of intron 2. Amplification of the \( fkbA \) coding domain sequence from a cDNA library synthesized from SM2 total RNA revealed the presence of several alternative \( fkbA \) cDNA species of various sizes (Fig. 5B). Sequence analysis of the smallest species revealed a 28-bp deletion of exon 3 sequence due to the use of a cryptic splice site located downstream from the acceptor splice site in wild-type \( fkbA \) (see Fig. S2 in the supplemental material). In two of the \( fkbA-1 \) mRNA species, intron 2 was retained due to the A316G substitution, leading to longer \( fkbA \) cDNA species (see Fig. S2). For all three cDNA/mRNA species, the predicted proteins contain premature stop codons upstream of the FK506 binding domain (see Fig. S2), and no FKBP12 protein products were detectable by Western blotting (Fig. 5C).

The FK506-resistant isolate SM4 contains an \( fkbA \) allele (\( fkbA-2 \)) in which a mutation in exon 3 results in a leucine-to-proline substitution (L91P) in the predicted protein sequence of FKBP12 that does not affect FKBP12 protein production (Fig. 5C). Leucine 91 is a highly conserved residue among FKBP12 homologs (Fig. 5D) and has been identified as a critical residue required for binding of human FKBP12 to calcineurin (21). In SM4, the L91P substitution leads to a shorter cyclic side chain that would predictably diminish calcineurin binding in the presence of FK506 (see Discussion). Taken together, our genetic analysis strongly suggests that FKBP12 functions as the rapamycin/FK506 receptor in \( M. \) circinelloides.

Disruption of \( fkbA \) by homologous recombination renders \( M. \) circinelloides resistant to rapamycin and FK506. To further establish that FKBP12 is the cognate receptor for rapamycin and FK506, we disrupted the \( fkbA \) locus by replacing the coding domain sequence with a pyrG disruption cassette. A gene replacement allele comprised of the pyrG gene and 1-kb sequences from the \( fkbA \) 5'- and 3'-untranslated regions (see Materials and Methods) was linearized and used to transform the uridine auxotrophic strain MU402 (\( \text{leuA}^+/H11002 \) \( \text{pyrG}^+/H11002 \)) (36). Two independent transformations were performed to ensure that disruption of the \( fkbA \) gene resulted from two independent events. PCR analysis of two independent homokaryotic uridine prototrophic transformants,
named RBM1 (fkbAΔ1) and RBM2 (fkbAΔ2), confirmed integration of the pyrG disruption allele at the fkbA locus (Fig. 6A and B). Disruption of fkbA in these two strains was also confirmed by Southern blot analysis (data not shown).

Northern analysis was further employed to confirm the loss of the fkbA gene. Employing a probe that hybridizes to exon 2 of fkbA, we were able to detect fkbA mRNA production in the wild-type parental strain (MU402) and failed to detect the message in each of the two independent fkbAΔ deletion strains (Fig. 6C). An antisense raised against S. cerevisiae FKBP12 (7) was employed to confirm the loss of the FKBP12 protein in both of the gene deletion strains. By Western analysis, we were able to detect a band of approximately 12 kDa (detected by comigration with appropriate size markers [data not shown]) that was not detected in the deletion strains (Fig. 6D). In the same Western blot, extracts from an S. cerevisiae wild-type strain (JK9-3da) and an fpr1Δ strain were included as positive controls for the anti-FKBP12 serum used (Fig. 6D).

After both fkbA deletion strains were rigorously verified, spores from each strain were germinated on YPD agar medium supplemented with rapamycin and FK506 in parallel with spores from the parental background strains, i.e., R7B and MU402 (MU402 was derived from R7B). While the growth of each parental strain was sensitive to rapamycin and FK506, growth of both fkbA mutant strains was unaffected in the presence of either drug. All strains were sensitive to growth inhibition in the presence of cyclosporine (Fig. 6E). Taken together, these results confirm that FKBP12, encoded by the fkbA gene, functions as the rapamycin/FK506 receptor in M. circinelloides.

**Rapamycin improves survival of Galleria mellonella larvae infected with a lethal dose of M. circinelloides spores.** Clinical treatment of human-invasive fungal infections caused by zygomycete (Rhizopus and Mucor) species is notoriously difficult to achieve due to the intrinsic resistance of most zygomycete species to the current antifungal drug armamentarium (reviewed in references 44 and 50). In light of the inhibitory effects of rapamycin on M. circinelloides growth, we tested whether targeting the Tor pathway with rapamycin could serve as a potential antifungal therapeutic approach. To this end, we used larvae from the wax moth Galleria mellonella infected with M. circinelloides spores as an invertebrate model of disseminated infection. The use of G. mellonella as a model of infection is becoming increasingly popular due to its ease of use and high correlation with murine models of disseminated infection (9, 12). Healthy larvae are light colored and active, while larvae killed by a fungal infection are dark and immobile, facilitating scoring of mortality during assays.
In this model of infection, injection of control PBS had no effect on survival of larvae (Fig. 7). Injection of 500 spores of the *M. circinelloides* R7B strain caused 100% death within 5 days after infection, while spores from the *M. circinelloides* NRRL3631 strain were avirulent in *G. mellonella* (Fig. 7), consistent with recent published results (31), and were not used in subsequent experiments.

Treatment of *G. mellonella* infected with *M. circinelloides* R7B spores with a dose of 33 mg of rapamycin/kg resulted in a 50% survival rate, a statistically significant (*P* < 0.0133; log rank test) improvement in survival compared to the 0% survival rate of PBS-treated infected controls (Fig. 8). Similar rapamycin treatment of *G. mellonella* larvae infected with spores from two independently derived R7B *fkbA* deletion strains (RBM1 and RBM2) did not have any effect on its virulence, indicating that inhibition of McTor by rapamycin is protective in *G. mellonella*. Notably, treatment of uninfected larvae with the same dose of rapamycin had a negligible effect on their survival in relation to PBS controls, indicating that rapamycin’s known immunosuppressive effect in humans has a negligible impact on *G. mellonella* survival (Fig. 8).

Surprisingly, and contrary to our *in vitro* results, monotherapy with FK506 did not improve survival of *G. mellonella* larvae infected with R7B spores at the dose administered (0.13 mg FK506/kg) (data not shown). This dose followed the MIC that elicited a growth inhibitory effect in our *in vitro* assays. However, FK506 proved not to be beneficial in the *in vivo* model of infection, possibly due to a reduced stability of FK506 in *G. mellonella*, higher rates of drug clearance, or competition for drug binding by endogenous FKBP12.

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Overall, the therapeutic benefit imparted by targeting the Tor pathway during infection of an invertebrate model of *M. circinelloides* dissemination establishes the Tor pathway as an attractive target for the development of antifungal therapy against life-threatening zygomycosis.

**DISCUSSION**

The Tor signaling pathway serves as an attractive target for antifungal therapy due to its central role in regulating fungal growth and its broad conservation among species in the fungal kingdom, with the notable exception of the Microsporidia (28, 47). The Tor signaling cascade evolved prior to the last common ancestor to the metazoan and fungal lineages, in accord with its known conservation with the deep ancestral roots of the Tor pathway, comparative genome analyses across multiple species throughout the fungal kingdom have revealed a remarkable conservation in the amino acid sequences of Tor homologs. Of particular interest are the invariably conserved residues within the hydrophobic pocket of the FRB domain that are critical for rapamycin binding to Tor (5, 7, 34, 47, 52, 59). This observation is in agreement with previous studies showing that rapamycin has broad-spectrum antifungal activity against several human pathogens, including *Candida albicans* and *Cryptococcus neoformans*, and that its activity in these species is mediated by conserved rapamycin-FKB12 drug-protein complexes (1, 13, 15, 17, 56, 58).

In this study, we further demonstrate that rapamycin inhibits growth of the zygomycetes *P. blakesleeanus*, *R. oryzae*, and *M. circinelloides*, demonstrating a conserved role for Tor in regulating the growth of basal fungal pathogens (Fig. 1 and Tables 2 and 3). We also established that rapamycin’s antifungal action is mediated via conserved FKB12-dependent inhibition of a Tor kinase homolog in *M. circinelloides*. First, expression of McFKBP12 in an *S. cerevisiae* FKB12-deficient strain restored rapamycin sensitivity (Fig. 4A). In addition, overexpression of the *M. circinelloides* Tor FRB domain rescued rapamycin toxicity in an *S. cerevisiae* wild-type strain (Fig. 4B). Rapamycin also promoted interactions between McFKBP12 and the McTor FRB domain in the yeast two-hybrid assay (Fig. 4C). These lines of evidence demonstrate a conserved mechanism of FKB12-mediated inhibition of Tor by rapamycin in a zygomycete fungal pathogen.

Second, we isolated a spontaneously rapamycin-FK506 doubly resistant mutant harboring a mutation in the acceptor splice site of intron 2 of *fkba* that abrogates FKB12 protein expression (Fig. 5A to C), presumably by nonsense-mediated decay. A second independent mutant, in which leucine 91 (a conserved calcineurin A binding residue in FKB12) is replaced by proline, while sensitive to rapamycin, displayed FK506 resistance (Fig. 5D). In the crystal structure of the human FKB12-FK506-calcineurin ternary complex, isoleucine 90 (I90) (corresponding to leucine 91 in *M. circinelloides* FKB12) changes conformation in the ternary complex and occupies a hydrophobic pocket in the calcineurin A subunit (21). Futer et al. identified I90 as a constituent of the human FKB12-FK506-calcineurin ternary complex, isoleucine 90 (I90) (corresponding to leucine 91 in *M. circinelloides* FKB12) changes conformation in the ternary complex and occupies a hydrophobic pocket in the calcineurin A subunit (21). Futer et al. identified I90 as a constituent of the human FKB12-FK506-calcineurin ternary complex, isoleucine 90 (I90) (corresponding to leucine 91 in *M. circinelloides* FKB12) changes conformation in the ternary complex and occupies a hydrophobic pocket in the calcineurin A subunit (21).
that targeting the Tor pathway with inhibitors could potentially be used during antifungal therapy against zygomycetes. These findings are in agreement with studies showing that rapamycin treatment protected 50% of mice from an otherwise lethal infection with \textit{C. albicans} and improved survival of mice with invasive aspergillosis (1, 26). Given that patients undergoing solid organ transplants are exceedingly susceptible to infections by zygomycetes, our findings suggest that patients receiving rapamycin immunosuppressive therapy may also benefit from its antifungal activities against infections by zygomycete pathogens. Indeed, studies have shown that solid organ transplant patients immunosuppressed with tacrolimus (FK506) have lower mortality rates for both cryptococcosis and zygomycosis (48, 49), warranting similar studies with patients immunosuppressed with rapamycin.

While the immunosuppressive effects of rapamycin currently outweigh its antifungal action, one report suggests that rapamycin in limited doses can synergize with antifungal agents against zygomycetes, providing a scenario in which rapamycin’s antifungal action might have therapeutic value. In one study (16), rapamycin was shown to exhibit synergistic activity with amphotericin B and posaconazole against several zygomycete clinical isolates, and it was shown to exhibit synergistic activity with amphotericin B and posaconazole against several zygomycete clinical isolates, and it showed antifungal activity on its own against Rhizopus microsporus, \textit{M. circinelloides}, \textit{Rhizomucor pusillus}, and \textit{Mucor circinelloides}. These results and our findings reported here provide support for harnessing the antifungal activity of rapamycin in combination with antifungals that are less effective against zygomycetes on their own.

Combinatorial therapy resulting in simultaneous inhibition of multiple pathways enhances the efficacy of individual drugs by broadening potency and decreasing the emergence of drug resistance. The challenge remains to exploit such combinatorial therapy while avoiding rapamycin’s immunosuppressive effects. Advances in this regard are under way with the development of less immunosuppressive rapamycin analogs with potent antifungal effects (14, 15, 38). Alternatively, possible clinical applications involve topical formulations, which circumvent the immunosuppressive effects, and the use of lipid formulations. Indeed, rapamycin encapsulated within PEG-DSPE (polyethylene glycol–1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy) micelles retains potent \textit{in vitro} activity against \textit{C. albicans} (54). Lipid formulations could also offer the advantage of masking immunosuppressive effects by allowing localized delivery of these drugs to sites of infections while also lowering effective doses and toxic side effects. Lipid formulations of rapamycin could potentially be beneficial in combinatorial therapy with lipid formulations of amphotericin B, the recommended course of treatment against zygomycosis (44, 51, 53). Hsp90 is also an attractive path for antifungal intervention in conjunction with Tor inhibitors, since Hsp90 inhibitors have robust antifungal activity against several fungal pathogens (12). Like the case with rapamycin, the challenge remains to develop specific inhibitors capable of discriminating pathogen protein chaperones from those of the host.

In conclusion, our studies have defined an evolutionarily conserved mechanism of rapamycin action in a basal fungal pathogen, a theme that is emerging as a broadly conserved paradigm in the fungal kingdom. It now remains to be tested whether rapamycin and less immunosuppressive analogs can be beneficial in vertebrate animal models of zygomycosis.

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