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Lovastatin Has Significant Activity against Zygomycetes and Interacts Synergistically with Voriconazole

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Zygomycetes are emerging opportunistic molds resistant to most conventional antifungals. We evaluated the in vitro activity of lovastatin (LOV), a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, against seven clinical isolates of *Zygomycetes* by using standard microdilution methods in three different media, disk diffusion testing, and viability dye staining. To further study the in vivo efficacy of LOV against zygomycetes, we developed a *Drosophila melanogaster* model of zygomycosis. In different experiments, groups of Toll-deficient (*Tl*) flies fed LOV-containing food were subsequently injected with two representative *Zygomycetes* isolates (*Mucor* and *Rhizopus* spp.). Finally, we examined the effects of LOV on voriconazole (VRC) activity against zygomycetes in vitro by checkerboard dilution, Epsilometer test-based methods, and bis-(1,3-dibutylbarbituric acid) trimethine oxonol staining and in vivo in *Tl* flies fed food containing LOV plus VRC and infected with zygomycetes. LOV exhibited significant, medium, and strain-independent fungicidal activity against all *Zygomycetes* isolates in vitro by all testing methods (MIC_{50}, 48.0 μg/ml; 50% minimal fungicidal concentration, 56.0 μg/ml; 50% effective concentration, 29.4 μg/ml [6.6 to 38.9 μg/ml]). *Tl* flies fed LOV-containing food and infected with *Mucor* had a significantly better 6-day survival rate than did infected *Tl* flies fed regular food ($P = 0.0005$). LOV displayed in vitro synergy with VRC against all *Zygomycetes* isolates (fractional inhibitory concentration index, 0.104 to 0.290) by all methods used. LOV also displayed synergy with VRC in the *Drosophila* model of zygomycosis ($P < 0.01$). LOV is significantly active against zygomycetes and synergizes with triazoles inherently resistant to them, such as VRC. The clinical significance of these findings needs to be further explored.

Fungi of the class *Zygomycetes*, order *Mucorales*, have been increasingly reported to cause opportunistic infections in a variety of immunocompromised hosts (11, 28). *Rhizopus* species cause more than 70% of *Zygomycetes* infections, whereas *Mucor*, *Absidia*, *Rhizomucor*, and *Cunninghamella* species are less frequently encountered pathogens (11, 16, 28). Importantly, zygomycosis has a particularly unfavorable outcome (11, 16, 28) because of delayed diagnosis as well as the inherent resistance of zygomycetes against most conventional antifungal agents (6, 11, 28, 30). Besides amphotericin B (AMB), only the investigational triazole posaconazole has shown promising activity against zygomycetes (6, 11, 28, 30, 32). Therefore, there is a dire need for development of novel treatment strategies against zygomycosis.

Lovastatin (LOV) is a statin that acts by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, which is the rate-limiting enzyme of the mevalonate pathway (26). LOV as well as other statins has activity against a variety of pathogens both in vitro and in vivo (12, 13, 31). Importantly, statins have been shown to have antifungal activity in vitro against both the nonpathogenic yeast *Saccharomyces cerevisiae* and the pathogenic yeasts *Candida* spp. and *Cryptococcus neoformans* (5, 22). In a recent study, LOV induced apoptosis-like cell death in a *Mucor racemosus* isolate at relatively high concentrations (29). Moreover, statins have been reported to exhibit synergistic interactions with azoles against *Candida*, even against azole-resistant *Candida* isolates (5).

In the present study, we evaluated the activity of LOV against a range of clinically important *Zygomycetes* spp. in vitro by using independent susceptibility methods. We also tested in a *Drosophila melanogaster* model of zygomycosis the efficacy of LOV in vivo against two representative *Zygomycetes* isolates (21). We further evaluated whether LOV and voriconazole (VRC), a triazole with an inherent lack of activity against *Zygomycetes* spp. (6, 11, 30), have synergistic effects in vitro and in vivo (*Drosophila* model) against zygomycetes. Both these pilot in vitro and in vivo studies against zygomycetes demonstrated that LOV has significant, broad-spectrum fungicidal activity and synergy with VRC.

MATERIALS AND METHODS

***Zygomycetes* isolates.** We used four *Rhizopus* isolates (two *Rhizopus homothallicus* isolates and two *Rhizopus oryzae* isolates), two *Mucor circinelloides* isolates, and one *Cunninghamella bertholletiae* isolate collected from patients with cancer who had zygomycosis. We confirmed genus identification of the *Zygomycetes* isolates as described previously (33). *Candida parapsilosis* strain ATCC 22019 was used for quality control purposes in all in vitro experiments.

Drug stock solutions. LOV (mevilonin) was kindly provided by Merck (Merck, Sharp, and Dohme Research Laboratories, Rahway, N.J.). LOV was hydrolyzed in active open acid form as described previously (9), and a stock solution (4 mg/ml in a 10% alcohol sterile solution) was stored at −20°C until use. Additionally, AMB deoxycholate (Sigma Chemical Co., St. Louis, Mo.), itraconazole (ITC) (Janssen Pharmaceutica, Titusville, N.J.), VRC (Pfizer, Inc., New York, N.Y.), and caspofungin (CAS) (Merck, Rahway, N.J.) were obtained in

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TABLE 1. Susceptibilities of the seven clinical isolates of *Zygomycetes* spp. to the antifungal agents tested in RPMI medium[a]

Isolate	MIC/MFC (μg/ml)			
	AMB	ITC	VRC	CAS
C. bertholletiae 506313	1.0/2.0	2/4	8/>16	>32/32
R. homothallicus 541783	0.5/1.0	4/4	8/>16	>32/32
R. oryzae 518749	0.5/1.0	4/8	16/>16	>32/32
R. homothallicus 529120	0.5/1.0	2/16	8/>16	>32/32
R. oryzae 557969	0.5/0.5	2/8	8/>16	>32/32
M. circinelloides 424760	0.5/1.0	4/16	8/>16	>32/32
M. circinelloides 488128	0.5/0.5	4/16	8/>16	>32/32

[a] CLSI broth microdilution method M-38A.

assay powder form. Drug stock solutions for each antifungal agent (1,280 μg/ml) were prepared in 100% dimethyl sulfoxide (for AMB and ITC) or double-distilled water (for all other agents).

Culture medium. The Clinical and Laboratory Standards Institute (CLSI) M38-A standard medium RPMI 1640 (Sigma Chemical Co.) was buffered with 0.165 M 3-(*N*-morpholino) propanesulfonic acid (MOPS) to pH 7 according to the manufacturer's instructions. Two other media were used to test the in vitro activity of LOV against the *Zygomycetes* isolates: RPMI medium plus 2% glucose (RPMI-2) and a yeast nitrogen base (YNB) medium (Difco, Detroit, Mich.).

CLSI susceptibility testing. The MICs of each antifungal agent and LOV against all of the *Zygomycetes* isolates were determined according to CLSI guidelines (M38-A document [27]). Standardized inoculum suspensions were prepared from 5- to 7-day-old cultures grown on potato dextrose agar slants, filtered twice through sterile syringes filled with glass wool, and adjusted with a hemacytometer to a concentration of 1×10^6 to 5×10^6 conidia/ml in sterile water. Conidial suspensions were further diluted 1:50 in RPMI medium, and 100 μl was dissolved in each well of a 96-well flat-bottomed microtitration plate (Corning, Inc., Corning, N.Y.) containing 100 μl of a serial twofold dilution of each antifungal agent. The final concentrations of the tested drugs ranged from 0.03 to 16.00 μg/ml for AMB, ITC, and VRC; 0.06 to 32.00 μg/ml for CAS; and 2.00 to 64.00 μg/ml for LOV. The MICs of LOV and each antifungal agent were determined at 24 h as the lowest drug concentration at which there was complete inhibition of growth. The minimal effective concentration of CAS was defined as the lowest drug concentration that resulted in the formation of aberrantly growing hyphal tips (3). All of the isolates were tested in triplicate on different days.

The minimum fungicidal concentration (MFC) of each antifungal agent was determined as described previously (8). Briefly, 20-μl suspensions from each well that showed complete inhibition of growth (100%) and from the last positive well (showing growth similar to that in the control well) were subcultured onto YNB plates prepared according to the manufacturer's instructions. The MFC was defined at 24 h as the lowest drug concentration at which fewer than three colonies were observed, which corresponded with a killing activity of approximately ≥99.9%. MFC determinations were done in triplicate on different days.

Disk diffusion susceptibility testing. We performed disk diffusion susceptibility testing on RPMI agar plates previously prepared by using standardized methods (20). Two hundred microliters of a standardized suspension of conidia (10^6 conidia/ml) of each *Zygomycetes* isolate was plated. After the plates were allowed to dry, a sterile one-quarter-inch paper disk (Schleicher and Schuell, Keene, N.H.) was placed on the agar surface and inoculated with 125 μl of LOV (4 mg/ml), producing a final LOV concentration in each plate of 20 μg/ml. Plates were incubated at 35°C, and the radius of the zone of inhibition was measured at 24 h by using a micrometer. AMB (final concentration, 10 μg/ml) was used as a control. Three independent experiments were performed at different time points.

XTT colorimetric assay. We performed the 2,3-bis[2-methyloxy-4-nitro-5-[(sulfenylamino) carbonyl]-2*H*-tetrazolium-5-carboxanilide] (XTT) colorimetric formazan reduction assay by using the method developed by Meletiadis et al. (25). We initially determined the relationship of formazan production to fungal inoculum by incubating standardized conidial suspensions of each isolate (10^2 to 10^5 conidia/ml) in RPMI medium for 12 h at 35°C. After 10 h of incubation, 50 μl of an XTT solution (1 mg/ml) containing 125 μg of menadione (Sigma Chemical Co.) was added to each well, and the tray was incubated for an additional 2 h. Formazan absorbance in each well was read at 492 nm and 690 nm (plate absorbance) with the use of a microplate spectrophotometer (Powerwave X; Biotech Instruments, Winooski, Vt.).

We performed XTT-based microdilution studies with CLSI microtiter plates prepared as described above. To evaluate the interaction of LOV with VRC against *Zygomycetes*, we performed two-dimensional (eight-by-eight) checkerboard studies into microtiter plates as previously described (19). The final concentrations of the drugs ranged from 2.00 to 64.00 μg/ml for LOV and 0.5 to 64.00 μg/ml for VRC. Wells were subsequently inoculated with 100 μl of a standardized conidial suspension of each *Zygomycetes* isolate (final concentration, 0.4×10^4 to 5.0×10^4 conidia/ml), and trays were incubated for 22 h at 35°C. Next, 50 μl of the XTT solution was added to each well, the tray was incubated for an additional 2 h, and formazan absorbance was determined as described above. The fractional inhibitory concentration (FIC) of each drug was calculated by dividing the MIC of the drug when used in combination by its MIC when used alone. FIC values then were added together to define the interaction of the combination (19). Synergy was defined as an FIC of ≤0.5, whereas antagonism was defined as an FIC of >4; off-scale MICs were raised to the next highest MIC. Control wells containing medium alone were included in each experiment. All experiments were performed in triplicate.

DiBAC morbidity staining of *Zygomycetes* isolates. Staining with the fluorescent dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC) was performed as described previously by Bowman et al. (3). Briefly, conidia from representative *Zygomycetes* isolates (one each of *R. oryzae*, *M. circinelloides*, and *C. bertholletiae*) were suspended in RPMI medium at a final concentration of 0.4×10^4 to 5.0×10^4 conidia/ml and incubated for 12 h at 35°C to generate hyphae. Aliquots of the hyphae were then mixed with each drug to produce the desired final concentration of LOV alone (4× 50% effective concentration [EC_{50}]), VRC alone (2 μg/ml), VRC in combination with LOV (2 and 4 μg/ml, respectively), and AMB (2 μg/ml). After incubation at 35°C for an additional 6 h, hyphae were washed twice in MOPS (pH 7) buffer solution. DiBAC stain (final concentration, 2 μg/ml) was added accordingly, and samples were incubated for 1 h at room temperature in the dark with shaking (3). Samples were then washed twice again with MOPS, pH 7, and resuspended for photomicrography. Photomicrographs of the hyphae were taken under a triple-band fluorescent microscope (Olympus BX-51; Olympus, Melville, N.Y.) as described previously (3).

Study of in vitro synergy of LOV and VRC by agar dilution and Epsilometer testing methods. To evaluate the effects of LOV on VRC activity against zygomycetes, we used a strategy combining the Epsilometer test (Etest; AB Biodisk, Solna, Sweden) and agar dilution susceptibility methods as described previously

TABLE 2. MICs and MFCs of LOV against the seven clinical isolates of *Zygomycetes* spp. in RPMI, RPMI-2, and YNB media[a]

Isolate	Result for LOV (μg/ml [±95% CI[b]])								
	RPMI			RPMI-2			YNB		
	MIC/MFC	EC_{50}	Hill slope	MIC/MFC	EC_{50}	Hill slope	MIC/MFC	EC_{50}	Hill slope
C. bertholletiae 506313	40/48	33.9 ± 6.6	−2.3 ± 1.1	32/32	7.3 ± 0.9	−4.9 ± 7.2	32/40	28.6 ± 5.4	−6.4 ± 7.0
R. homothallicus 541783	56/56	38.9 ± 7.2	−4.7 ± 3.7	56/56	55.8 ± 52.0	−1.5 ± 2.2	48/64	30.6 ± 27.2	−1.2 ± 2.3
R. oryzae 518749	48/48	27.1 ± 13	−3.3 ± 5.9	40/48	20.4 ± 12.5	−1.3 ± 1.6	32/40	19.2 ± 10.3	−1.5 ± 1.0
R. homothallicus 529120	48/56	20.2 ± 3.1	−4.0 ± 2.4	56/56	17.7 ± 2.6	−4.3 ± 3.0	40/64	19.4 ± 4.0	−1.9 ± 0.9
R. oryzae 557969	56/64	32.9 ± 2.4	−5.5 ± 1.9	48/64	35.9 ± 11.6	−1.2 ± 0.7	56/64	11.3 ± 9.7	−0.8 ± 0.7
M. circinelloides 424760	32/32	6.6 ± 2.5	−1.2 ± 0.6	16/24	6.2 ± 2.5	−1.8 ± 1.5	24/24	9.8 ± 3.5	−2.4 ± 2.3
M. circinelloides 488128	56/56	29.4 ± 4.4	−3.8 ± 2.3	48/48	27.0 ± 3.0	−4.4 ± 1.8	40/40	24.7 ± 6	−2.4 ± 1.3

[a] CLSI broth microdilution method M-38A.
[b] CI, confidence interval.

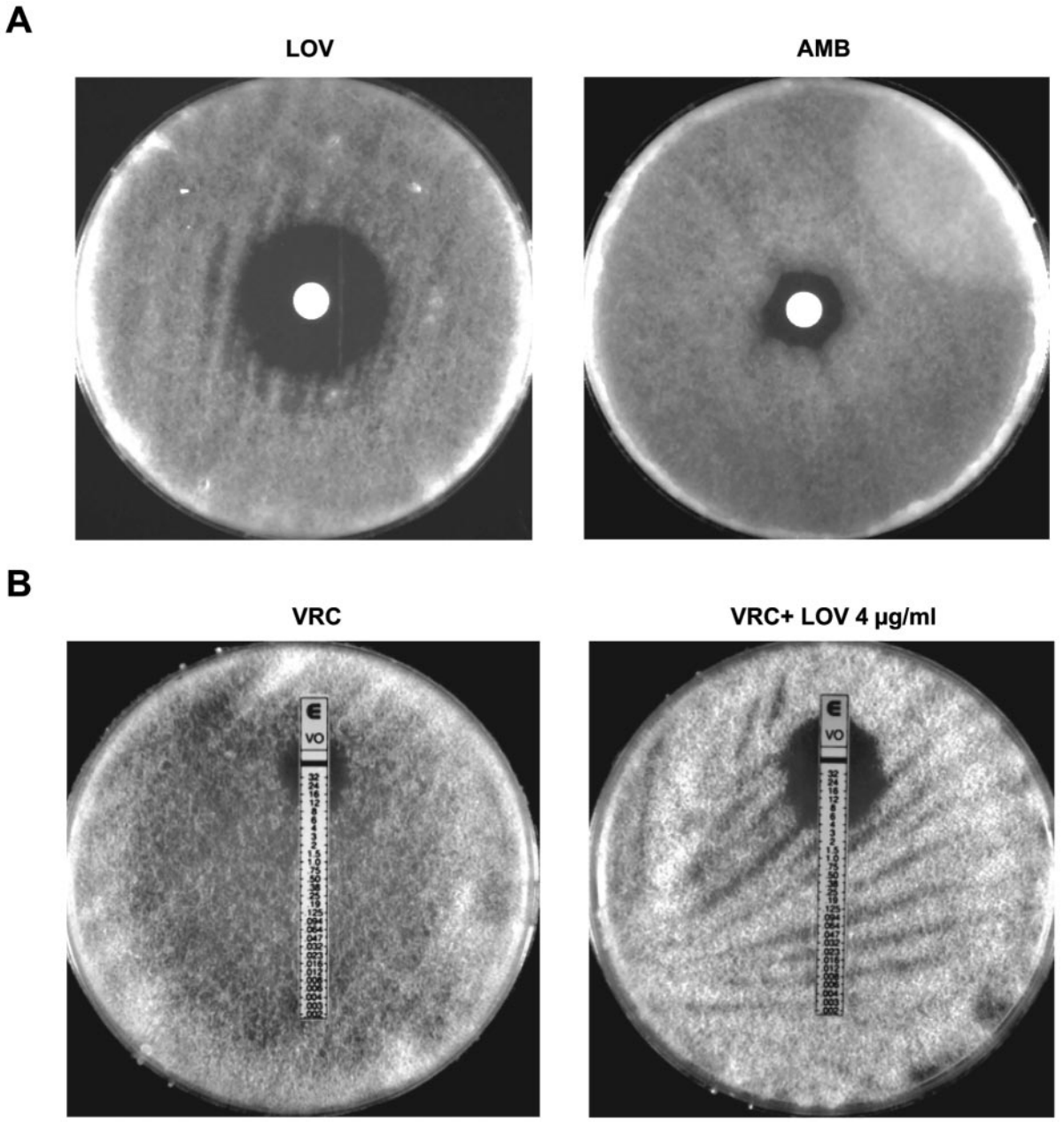

FIG. 1. (A) Effects of LOV compared with effects of AMB against a representative *Zygomycetes* isolate (*M. circinelloides* 424760) as seen with disk diffusion susceptibility testing. Each disk contained 125 μl of LOV, resulting in a final LOV concentration in each plate of 20 μg/ml from a stock solution of 4 mg/ml, and 50 μl of AMB, resulting in a final AMB concentration in each plate of 10 μg/ml from a stock solution of 5 mg/ml. (B) Change in the VRC MIC against a representative *Zygomycetes* isolate (*M. circinelloides* 424760) with simultaneous exposure to LOV as assessed by Etest susceptibility testing. Each *Zygomycetes* isolate was tested on regular RPMI agar plates and on RPMI agar plates containing a noninhibitory concentration of LOV (4 μg/ml). All of the experiments were performed in triplicate on different days.

(15). We performed Etest susceptibility testing of VRC against each *Zygomycetes* isolate in RPMI plates containing a standard noninhibitory concentration of LOV (4 μg/ml) (7). As controls, we determined VRC MICs against *Zygomycetes* by using the Etest method on regular RPMI plates (without LOV). We read the VRC MIC at 24 h as the drug concentration at the point where the growth ellipse intersected the strip (7) and, accordingly, statistically compared the changes in VRC MICs (15). All MIC determinations were carried out in triplicate on different days; the median 24-h MICs were reported.

***Drosophila* infection model.** In different experiments, we infected *Toll*-deficient flies (*Tl* flies) (2- to 4-day-old female flies, 30 per experimental group) with two representative *Zygomycetes* isolates (*M. circinelloides* 424760 and *R. oryzae* 557969). We injected the thoraxes of *Tl* flies with a thin sterile needle that had been dipped into a concentrated solution (10^8 conidia/ml) of each *Zygomycetes* isolate, as described previously (17, 20). After injection, we housed the flies at 29°C and transferred them to fresh vials every 2 days. We assessed survival daily until day 6 after injection. We performed each experiment at least in triplicate on different days.

Drug protection experiments. For assessment of LOV protection against lethal infection by each *Zygomycetes* isolate, different groups of *Tl* flies (30 per experimental group) were housed in empty vials for 6 to 8 h to starve and then transferred into vials containing LOV-mixed fly food (10 mg/ml) as described previously (2, 20). After 48 h, each group of *Tl* flies was infected with the

corresponding *Zygomycetes* isolate by injection and transferred daily into fresh LOV-containing vials at 29°C for 6 days. Flies that were starved for 6 to 8 h, transferred to vials containing regular fly food (without LOV), infected, and maintained in regular vials were used as controls. LOV protection was assessed daily until day 6 after infection. For the combination drug experiments, vials containing VRC alone (1 mg/ml), LOV alone (10 mg/ml), or VRC plus LOV (1 and 10 mg/ml, respectively) were prepared as described previously (20). Each experiment was performed at least in triplicate on different days.

Statistical analysis. Median MIC and MFC values were calculated based on experiments performed in triplicate. The Mann-Whitney U test or Kruskal-Wallis one-way analysis of variance with Dunn's test was used when appropriate to assess significant differences in the corresponding MICs and MFCs. Survival curves were plotted by using Kaplan-Meier analysis, and differences in survival rates between the groups were analyzed by using the log rank test. A four-parameter logistic regression model (Hill equation) was fitted to XTT reduction assay data to determine EC_{50} values and the steepness of inhibitory dose-response curves (Hill slope) with the use of a curve-fitting software program (Prism 4; GraphPad Software, Inc., San Diego, Calif.). *P* values of less than 0.05 were considered statistically significant.

RESULTS

LOV has significant in vitro activity against *Zygomycetes* isolates. The MICs and MFCs for AMB, ITC, VRC, and CAS are listed in Table 1. AMB exhibited fungicidal activity against all of the *Zygomycetes* isolates tested, as the AMB MICs and MFCs (MIC_{50}, 0.5 μg/ml; MFC_{50}, 1 μg/ml) were equal or differed by less than 1 serial dilution in every case. By comparison, only ITC (MIC_{50}, 4 μg/ml; MFC_{50}, 8 μg/ml) demonstrated limited fungistatic efficacy against some *Zygomycetes* isolates, whereas neither VRC (MIC_{50}, 8 μg/ml; MFC_{50}, 16 μg/ml) nor CAS (MIC_{50}, 32 μg/ml; MFC_{50}, 32 μg/ml) had any activity at the concentrations tested.

The MICs and MFCs of LOV against *Zygomycetes* isolates in RPMI, RPMI-2, and YNB media are shown in Table 2. LOV exhibited fungicidal activity against all of the isolates (MIC_{50}, 48 μg/ml [range, 32 to 56 μg/ml]; MFC_{50}, 56 μg/ml [range, 48 to 64 μg/ml]) in all media tested. Although there were no significant interspecies differences, there was a trend towards improved activity of LOV against *Mucor* species.

The activity of LOV against zygomycetes was also clearly observable with disk diffusion susceptibility testing (Fig. 1A). Notably, LOV produced a clear zone of inhibition of growth against all *Zygomycetes* isolates (mean radius ± standard deviation for LOV, 12.570 ± 3.861 mm).

LOV results in significant, concentration-dependent reduction of *Zygomycetes* biomass by the XTT assay. We found a linear relationship between XTT reduction to formazan (the colorimetrically assayed product) and starting inoculum of zygomycetes for all of the isolates tested over a range of inocula (Fig. 2A). Therefore, a reduction of the formazan absorbance from 3.0 to ≤0.5 optical density as a result of LOV activity against zygomycetes correlated with an approximately-2.5-log reduction in hyphal biomass.

LOV exhibited potent antifungal activity against all of the *Zygomycetes* isolates (median EC_{50}, 29.4 μg/ml [range, 6.6 to 38.9 μg/ml]) in a steep concentration–inhibitory-effect curve (Hill slope range, −1.2 to −4.7). A substantial reduction (≥2.5 log) of the hyphal biomass was evident for all of the isolates as the LOV concentration approached the MFC (Fig. 2B). Again, LOV appeared to be more effective against *Mucor* spp. than against *Rhizopus* and *Cunninghamella* spp. The in vitro activity of LOV was consistent among all three media tested (Table 2).

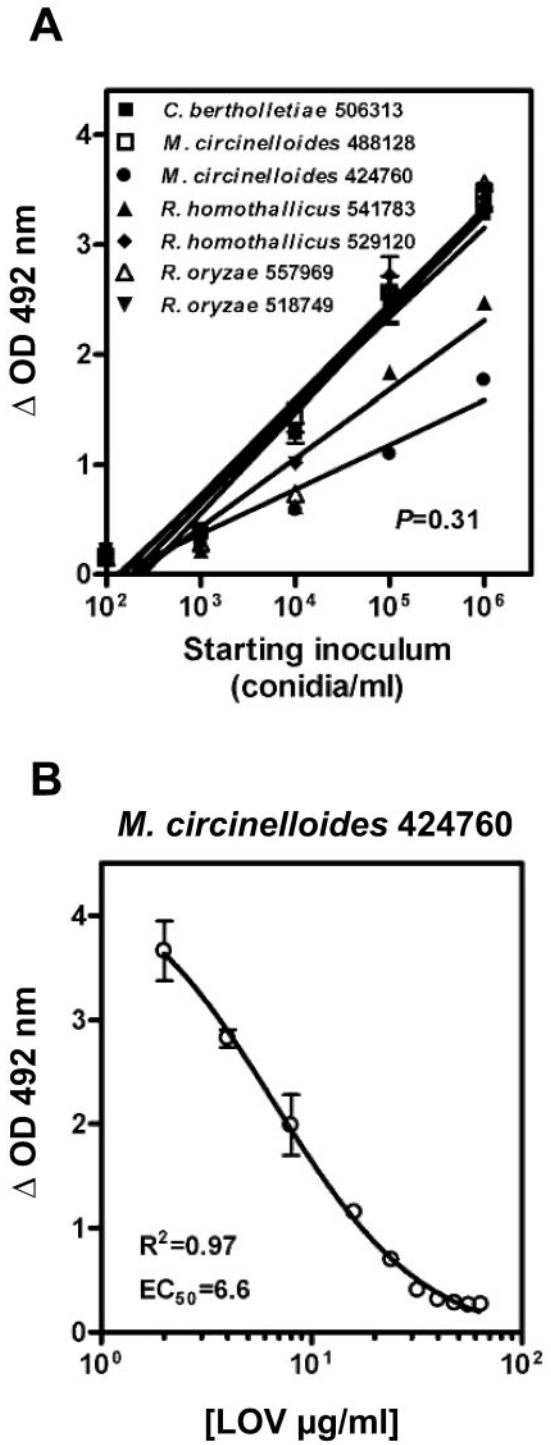

FIG. 2. (A) Regression plots of the relationship between fungal inoculum and formazan production for all of the *Zygomycetes* isolates at 12 h. The slopes of the regression plots ranged from 0.62 to 0.95 and were not significantly different by analysis of variance ($P = 0.31$). The coefficients of determination (R^2) were high for all of the tested isolates (range, 0.89 to 0.96). (B) XTT-based analysis of the in vitro activity of LOV in RPMI media against a representative *Zygomycetes* isolate (*M. circinelloides* 424760). Sigmoid concentration–inhibitory-effect curves were generated by fitting data to a four-parameter logistic regression model (Hill equation). The symbols represent the means ± standard deviations for experiments performed in triplicate in each case. OD, optical density.

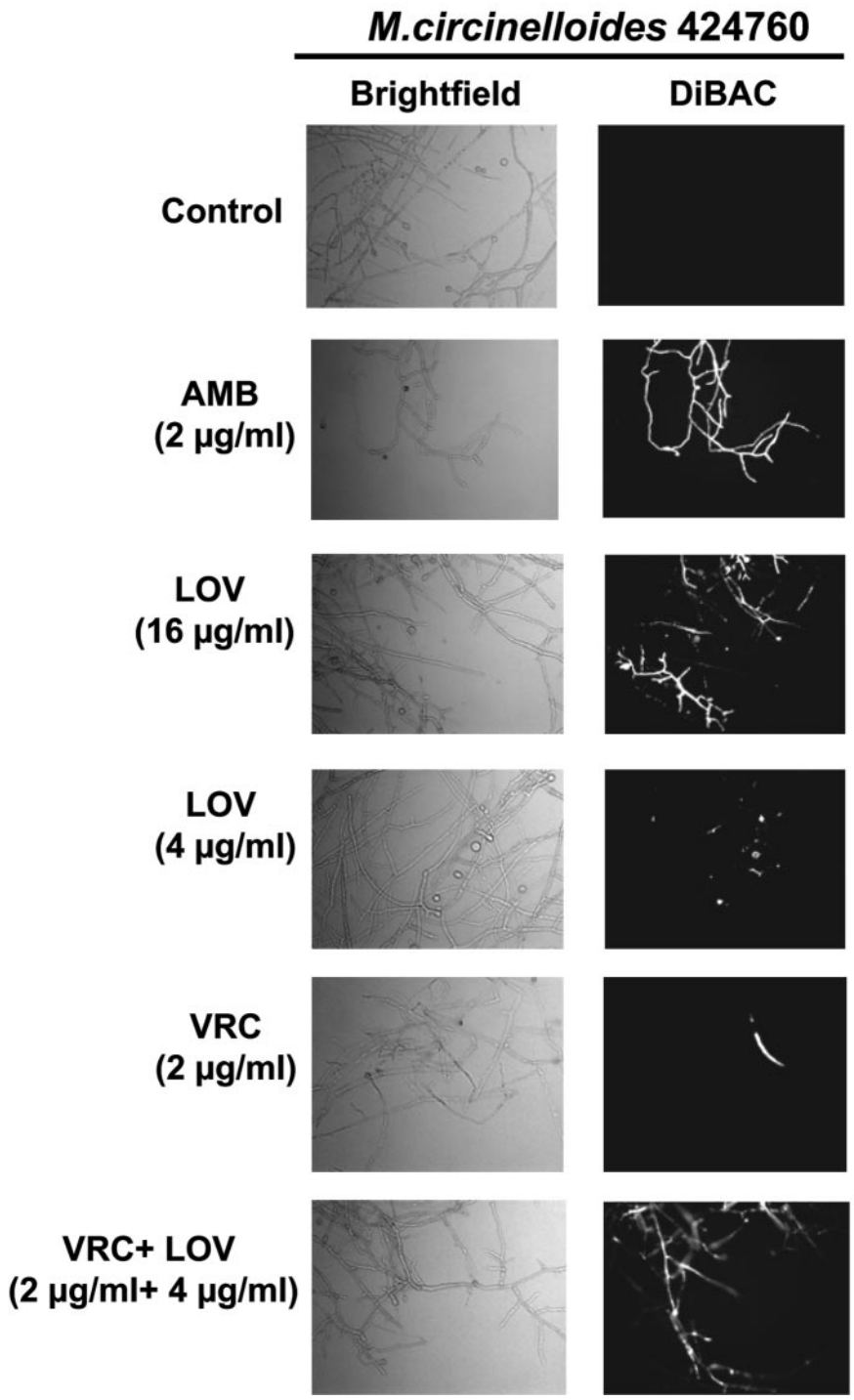

FIG. 3. Detection of hyphal damage to the representative isolate *M. circinelloides* 424760 by fluorescent microscopy with the cellular morbidity dye DiBAC following exposure to antifungals. *Zygomycetes* hyphae were prepared after 18 h of incubation in RPMI media, washed, and resuspended in RPMI media containing AMB (2 μg/ml), LOV (16 and 4 μg/ml), VRC (2 μg/ml), or VRC in combination with LOV (2 and 4 μg/ml, respectively). Untreated hyphae were used as controls. After 6 h of incubation, cells were washed and stained with DiBAC. Hyphae were then examined with the use of bright-field (light boxes) and epifluorescence (black boxes) microscopy at ×400 with Normanski optics and a fluorescein isothiocyanate filter. The fluorescence in the dark boxes is indicative of early hyphal damage by the corresponding antifungal agent.

LOV has fungicidal effects against *Zygomycetes* hyphae by DiBAC staining. While control hyphae cells (without drug preexposure) demonstrated absence of fluorescence after DiBAC staining, there was clearly evident *Zygomycetes* hyphal damage in high concentrations of LOV (4× EC_{50}; 16 μg/ml) of each isolate tested; at a subinhibitory LOV concentration (4 μg/ml), the fluorescence indicative of hyphal damage caused by LOV was minimal to absent (Fig. 3). AMB exhibited a pronounced

hyphal damage at a concentration equal to the MFC (2 μg/ml), whereas VRC had no fungicidal activity at a clinically achievable concentration (2 μg/ml).

Protection of *Tl* flies infected with *Zygomycetes* by LOV. Injection of a concentrated solution of conidia (10^8 conidia/ml) of either *R. oryzae* or *M. circinelloides* resulted in a hyperacute infection with equally high mortality rates in *Tl* flies (>80%) within 3 days of infection (Fig. 4A). LOV-fed flies had a significantly better survival rate 6 days after infection with *M. circinelloides* (50%) than did control flies (<15%; $P = 0.0005$) (Fig. 4A). However, LOV had no activity in *Tl* flies infected with *R. oryzae* (Fig. 4A).

LOV exhibits synergistic interaction with VRC in vitro when tested in combination against *Zygomycetes* isolates. By XTT-based checkerboard, the combination of both LOV and VRC was synergistic against all of the *Zygomycetes* isolates tested (FIC index of ≤0.5), with FIC indices ranging from 0.104 to 0.290 (Table 3). Importantly, the interaction of LOV with VRC resulted in MICs (VRC MIC_{50}, 1 μg/ml; LOV MIC_{50}, 2 μg/ml) that were in the range of clinically achievable concentrations of both drugs (Table 3).

LOV and VRC have significant synergistic effects against *Zygomycetes* by Etest/plate dilution method. The effects of LOV on VRC MICs against all of the *Zygomycetes* isolates by Etest/plate dilution method are shown in Table 3 and Fig. 1B. There was a significant, pronounced reduction in VRC MICs (threefold to sixfold) following concomitant exposure of all of the *Zygomycetes* isolates to both LOV and VRC ($P < 0001$).

LOV combination with VRC is fungicidal against *Zygomycetes* by DiBAC staining. The combination of VRC and LOV at subinhibitory concentrations for both drugs (2 and 4 μg/ml, respectively) potentiated fungicidal activity against *Zygomycetes* hyphae, as evidenced by DiBAC staining (Fig. 3). In pilot experiments, VRC alone was shown to cause minimal fluorescence indicative of cellular wall and membrane damage in *Zygomycetes* hyphae at concentrations up to 8 μg/ml. The effects of VRC combined with LOV on hyphal damage were comparable with those of AMB at concentrations equal to the MFC (2 μg/ml).

Protection of *Tl* flies infected with *M. circinelloides* by the combination of LOV and VRC. Previous studies showed that concentrations of VRC up to 2 mg/ml were not toxic in adult flies (21). We found that *Tl* flies fed with LOV plus VRC and infected with either *R. oryzae* or *M. circinelloides* had a significantly better survival rate than did infected flies fed with LOV alone ($P < 0.01$). VRC alone had no activity in infected *Tl* flies (Fig. 4B).

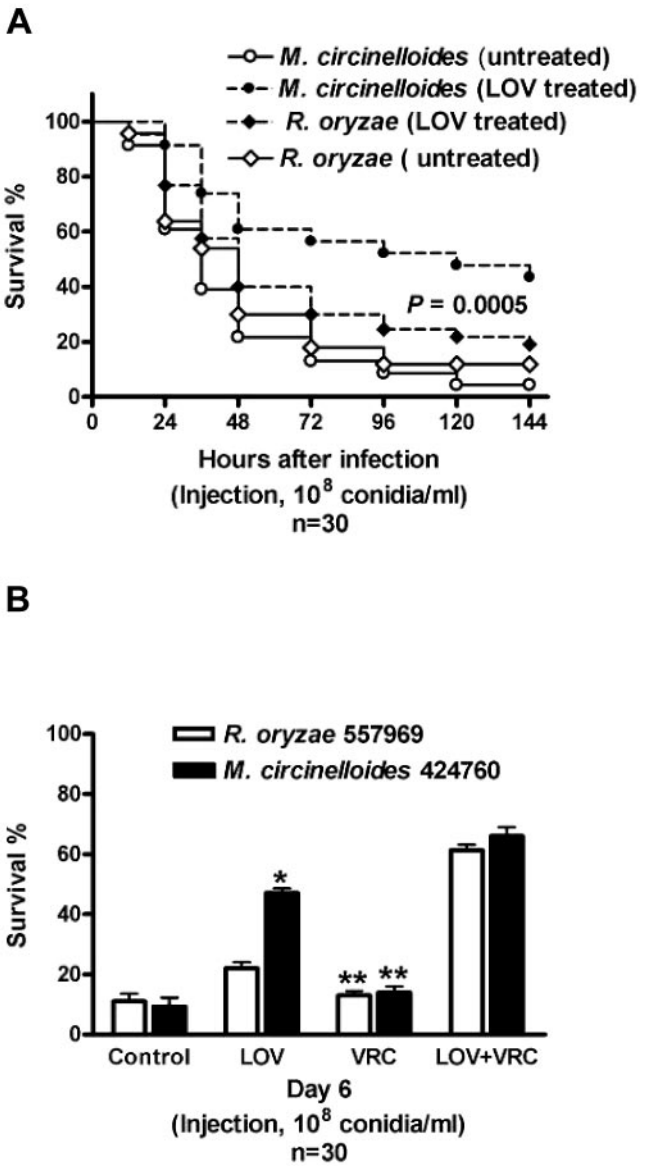

FIG. 4. (A) LOV protection of *Tl* flies infected by injection of each representative *Zygomycetes* isolate (*M. circinelloides* 424760 or *R. oryzae* 557969; 10^8 conidia/ml). Survival curves of LOV-treated and untreated (control) flies are shown. The data represent the means of four independent experiments (30 flies per group). The *P* value was <0.0005 for LOV-treated flies infected with *M. circinelloides* versus control (untreated flies), and the *P* value was not significant for LOV-treated flies infected with *R. oryzae* versus control (untreated flies). (B) Survival of *Tl* flies treated with VRC alone, LOV alone, or VRC plus LOV 6 days after infection by injection of each representative *Zygomycetes* isolate (*M. circinelloides* 424760 or *R. oryzae* 557969; 10^8 conidia/ml). The data represent the means of four independent experiments (30 flies per group), and the bars represent the standard deviations. ∗, *P* of <0.01 for VRC plus LOV versus LOV alone; ∗∗, *P* of <0.001 for VRC plus LOV versus VRC alone.

DISCUSSION

The development of oral antifungal therapeutic strategies is a major unmet medical need for zygomycosis, an opportunistic mycosis for which oral antifungal options are extremely limited (11). In the present study, we found that the in vitro susceptibilities of the *Zygomycetes* isolates to the antifungal drugs tested were in agreement with those from previous studies (6, 11, 30). Indeed, of the antifungal agents tested, only AMB exhibited significant fungicidal activity. Although it has been reported that *Zygomycetes* spp. might be susceptible to ITC in vitro (6), ITC exhibited marginal fungistatic activities against the isolates tested in our study. However, the *Rhizopus*, *Mucor*, and *Cunninghamella* isolates that we tested were more resistant to ITC than were *Absidia* and *Rhizomucor* isolates tested elsewhere (6, 30). Our study also confirmed the well-described in vitro resistance of *Zygomycetes* spp. to VRC and CAS (6, 11, 30).

TABLE 3. In vitro interaction between VRC and LOV against *Zygomycetes* isolates[a]

Isolate	Result for:					
	XTT-based checkerboard				Etest/agar dilution[d]	
	MIC (μg/ml)			Lowest ΣFIC for VRC/LOV (interpretation)[c]	MIC (μg/ml)	
	VRC	LOV[b]	VRC/LOV		VRC	VRC/LOV
C. bertholletiae 506313	8	48	1/2	0.175 (S)	16	3
R. homothallicus 541783	8	56	1/2	0.155 (S)	32	12
R. oryzae 518749	16	48	1/2	0.104 (S)	32	8
R. homothallicus 529120	8	48	0.5/2	0.104 (S)	24	6
R. oryzae 557969	8	56	2/2	0.290 (S)	32	8
M. circinelloides 424760	16	32	1/2	0.125 (S)	12	4
M. circinelloides 488128	8	56	1/4	0.132 (S)	32	4

[a] Assessed by using XTT-based checkerboard and Etest/agar dilution susceptibility methods.
[b] The following LOV concentrations were used in checkerboard dilution studies: 2, 4, 8, 16, 32, 48, 56, and 64 μg/ml.
[c] S, synergy.
[d] $P = 0.0006$. MICs of VRC against *Zygomycetes* spp. by Etest/agar dilution method are given as results for VRC alone versus results for VRC in combination with LOV (4 μg/ml).

We also found that LOV had considerable in vitro fungicidal activity against all seven *Zygomycetes* isolates tested. Although we did not observe dramatic interspecies differences in susceptibility to LOV, the *Mucor* isolates appeared to be more susceptible to LOV than the other isolates were. In comparison, a recent study that used nonstandardized methods found that *Rhizomucor pusillus* strains were remarkably more sensitive to LOV than *Rhizomucor miehei* strains were according to agar diffusion susceptibility testing (23).

Furthermore, we observed that the activity of LOV against *Zygomycetes* isolates was consistent across several different culture media. Of note, there was a trend toward lower LOV MICs and EC_{50}s in YNB media. Our group and others have postulated that because enriched YNB medium facilitates fungal growth compared with RPMI and RPMI-2, it might result in increased metabolic activity of fungal cells and better drug penetration into the intracellular sites of drug action (20, 24).

To better characterize the effects of LOV against *Zygomycetes* spp., we employed a strategy combining different viability dye staining methods (18). We found that the XTT colorimetric assay was able to accurately quantify the *Zygomycetes* fungal biomass. However, the incubation period was much shorter than with other filamentous fungi (18). In agreement with studies of other filamentous fungi (18, 25), we found that the LOV EC_{50}s correlated better with the MICs for *Zygomycetes* spp.

We then microscopically confirmed the fungicidal activity of LOV against *Zygomycetes* spp. by staining LOV-treated hyphae with the fluorescence morbidity dye DiBAC. In correlation with XTT-based analysis, DiBAC staining showed that LOV had minimal fungicidal activity at a low concentration (2 μg/ml), whereas it caused prominent hyphal damage at fungicidal concentrations (4× EC_{50}; 16 μg/ml). The fungicidal effects of LOV were comparable with those of AMB at a fungicidal concentration (2 μg/ml).

To examine whether LOV has the same activity against *Zygomycetes* spp. in vivo, we adapted an established mini-host model of zygomycosis in *Drosophila melanogaster*. Importantly, this model organism has been successfully used to assess the in vivo effects of LOV in the field of neurological research (2). We found that LOV had a significant protective effect in flies infected with a *M. circinelloides* strain. However, LOV had no activity in *Tl* flies infected by a less susceptible in vitro *R. oryzae* strain. Although *Drosophila* offers several advantages over conventional, logistically more difficult animal models in the screening of candidate compounds for antifungal activity because of its simplicity and rapidity, it does not allow for quantification of orally absorbed drugs. As a result, pharmacodynamic and pharmacokinetic studies with *Drosophila* are challenging, whereas little is known of the metabolism of LOV in this model. Thus, our promising findings of LOV activity in *Tl* flies will need further validation with mammalian models of zygomycosis.

As statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a rate-limiting enzyme in ergosterol biosynthesis (26), azoles block a sequential target in the ergosterol biosynthetic pathway (C-14 demethylation) (22). We analyzed the interaction of LOV with VRC, a triazole with no meaningful activity against *Zygomycetes* spp., because the potential of statins to overcome azole resistance has been shown previously for *Candida* (5) and might have even greater clinical significance. By employing checkerboard dilution and Etest-based methods, we found that at subinhibitory concentrations, LOV significantly potentiated the activity of VRC against all of the *Zygomycetes* isolates tested in vitro. We further verified this observation by using DiBAC staining. Importantly, the concentrations of LOV used in synergy studies with VRC were within the range of serum concentrations of LOV reported for mammalian models (2 to 20 μM or 0.5 to 5 μg/ml) and for humans (3.9 μM or 1 μg/ml) (4). Finally, we found that LOV and VRC exhibited synergistic effects against two representative *Zygomycetes* isolates in vivo in the *Drosophila* model of zygomycosis. These results are in agreement with studies that reported in vitro synergy of statins with different azoles against *Candida* and *Cryptococcus* species (5). Importantly, there is also evidence of in vivo synergy of LOV and azoles against *Trypanosoma cruzi*, a protozoan parasite in which, similar to fungi, ergosterol is an essential cell membrane component (31).

Although sequential inhibition of sterol biosynthesis may be plausible (22), the mechanisms of the antifungal activity of LOV remain largely unexplored because of the pleiotropic effects of LOV in cellular metabolism. For example, LOV is a well-known inhibitor of protein isoprenylation, a highly conserved essential process for cell proliferation, differentiation,

and apoptosis in eukaryotes from fungi to humans (4, 10, 26) as well as in prokaryotes (34). Importantly, LOV was recently shown to induce apoptosis of several human cancer cell lines by mechanisms involving inhibition of *Ras* and several other isoprenoids (1, 4, 13, 14, 26). Similarly, LOV was recently shown to suppress the expression of three *Ras* genes in *M. racemosus* and to induce apoptosis-like cell death (29). However, the exact mechanisms that mediate apoptosis in fungi seem to be complex and have not been elucidated. Exploring the underlying mechanisms of LOV activity against *Zygomycetes* might pave the way for the development of compounds with more selective action. This is particularly important in view of the fact that significant toxicity has been reported as a result of statin interactions with azoles or other drugs that are metabolized in the liver by cytochrome P450 (4). Finally, expanding these observations to other members of the statin family with or without other azoles that exhibit activity against *Zygomycetes* (e.g., posaconazole) or other classes of antifungal agents would be of interest.

In conclusion, the significant antifungal activity of LOV against *Zygomycetes* as well as its synergy with triazoles may result in significant clinical applications in the near future.

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