

## In Vitro Interactions between Antifungals and Immunosuppressive Drugs against Zygomycetes<sup>∇</sup>

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**The in vitro interaction of antifungals with immunosuppressive drugs was evaluated against zygomycetes. The combination of amphotericin B with cyclosporine, rapamycin, or tacrolimus was synergistic for 90%, 70%, and 30% of the isolates, respectively. For posaconazole, itraconazole, and ravuconazole, synergy was more frequently observed with cyclosporine than with rapamycin or tacrolimus and antagonistic interactions were rarely noted. In summary, calcineurin inhibitors and rapamycin can be synergistic in vitro with amphotericin B and azoles against zygomycetes.**

Calcineurin inhibitors (cyclosporine and tacrolimus) and inhibitors of the mTOR pathway (rapamycin) are widely used with solid organ transplant (SOT) patients. Although the use of these immunosuppressive drugs is a risk factor for invasive fungal infections, they also possess antifungal activity against yeasts (3, 4, 36, 39) and filamentous (34) and dimorphic fungi (14). Nevertheless, the activity of immunosuppressive drugs alone or in combination against zygomycetes has been poorly evaluated. The combination of immunosuppressive drugs with antifungal drugs has been evaluated against yeasts (5, 7, 15, 17–21, 25, 26, 37, 38) and filamentous fungi (16, 27, 33, 34). Because zygomycosis is emerging in SOT patients, we studied combinations between amphotericin B or azoles and immunosuppressive agents against zygomycetes.

Combinations were tested against 10 isolates (3 *Rhizopus oryzae*; 2 *Mycocladius corymbiferus*, formerly *Absidia corymbifera* [11]; 2 *Mucor circinelloides*; 2 *Rhizopus microsporus*; and 1 *Rhizomucor pusillus*) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (2) modified for a broth microdilution checkerboard procedure. Final concentrations were 0.004 to 2 µg/ml for amphotericin B, itraconazole, posaconazole, and ravuconazole; 0.125 to 8 µg/ml for cyclosporine; and 0.06 to 4 µg/ml for tacrolimus and rapamycin. Lower concentrations of 0.001 to 0.06 µg/ml were tested for tacrolimus and rapamycin in additional experiments. More than 70% of the tests were run in duplicate and yielded identical ( $\pm 1 \log_2$  dilution) MICs between runs in more than 90% of the cases, confirming that the technique was reproducible. The MICs were determined spectrophotometrically after 24 h of incubation at 35°C. MIC endpoints were defined as the lowest drug concentration (tested alone or in combination)

that gave 50% of the inhibition except for amphotericin B alone, for which a complete inhibition (as measured by a 90% reduction of growth by spectrophotometric reading) was used. Calculations with two alternative sets of inhibition endpoints ([i] 90% for azoles and 50% for immunosuppressive drugs alone and for all combinations and [ii] 90% for all drugs either alone or in combination) were also performed. Antifungal combinations of amphotericin B or posaconazole with cyclosporine were also evaluated by Etest in RPMI agar after 24 h of incubation at 35°C. Drug combinations were evaluated by the calculation of the fractional inhibitory concentration indices (8) interpreted as previously recommended (24).

All isolates exhibited low MICs for amphotericin B (0.125 to 1 µg/ml). Itraconazole, posaconazole, and ravuconazole showed activity against most of the isolates, with geometric mean MICs of 0.62, 0.57, and 0.44 µg/ml, respectively (data not shown). Immunosuppressive drugs alone also demonstrated activity. Cyclosporine MICs varied between 1 and >8 µg/ml.

TABLE 1. Summary of the in vitro interaction of amphotericin B and azoles in combination with immunosuppressive drugs against 10 isolates of *Zygomycetes*

Combination <sup>a</sup>	Interaction (% of isolates)		
	Synergy	No interaction	Antagonism
AMB + CyA	90	10	0
AMB + Tacro	30	70	0
AMB + Rapa	70	30	0
ITZ + CyA	70	30	0
ITZ + Tacro	20	80	0
ITZ + Rapa	50	30	20
PSZ + CyA	70	30	0
PSZ + Tacro	0	100	0
PSZ + Rapa	40	60	0
RVZ + CyA	30	70	0
RVZ + Tacro	10	90	0
RVZ + Rapa	30	60	10

<sup>a</sup> AMB, amphotericin B; ITZ, itraconazole; PSZ, posaconazole; RVZ, ravuconazole; CyA, cyclosporine; Tacro, tacrolimus; Rapa, rapamycin.

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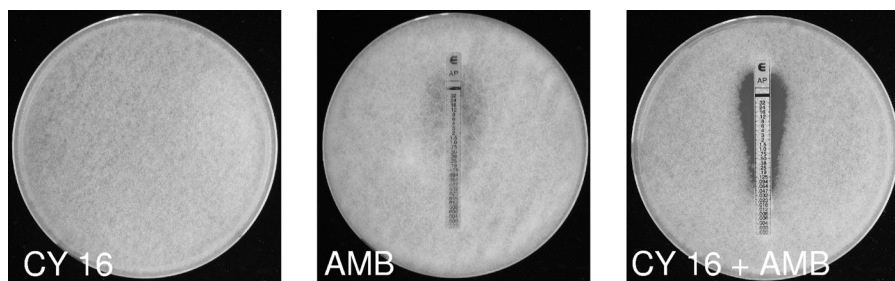


FIG. 1. In vitro interaction of amphotericin B (AMB) with cyclosporine (CY 16) against an isolate of *Rhizopus oryzae* (CNR 03\_918) tested by an agar diffusion method. Cyclosporine was incorporated in the agar. The amphotericin B MIC was determined by Etest in the absence or presence of cyclosporine at a concentration of 16  $\mu\text{g/ml}$ .

For tacrolimus and rapamycin, susceptibility depended on the species. Tacrolimus MICs were  $\leq 0.5$   $\mu\text{g/ml}$  for *R. oryzae*, *M. circinelloides*, and *M. corymbiferus* and  $>4$   $\mu\text{g/ml}$  for *R. microsporus* and *R. pusillus*. For rapamycin, *R. microsporus* and *M. circinelloides* exhibited MICs of  $\leq 0.25$   $\mu\text{g/ml}$ , whereas *R. pusillus* and *M. corymbiferus* had MICs of  $>4$   $\mu\text{g/ml}$ . For the three *R. oryzae* isolates, variable susceptibilities to rapamycin were observed.

The results of the 12 combinations tested are summarized in Table 1. Synergistic interactions were observed between amphotericin B and cyclosporine (90%), rapamycin (70%), and tacrolimus (30%). For the combinations including azoles, synergy was more frequently observed with cyclosporine (up to 70% for itraconazole and posaconazole) than with rapamycin or tacrolimus. Antagonism was never observed with amphotericin B or posaconazole in combination with any immunosuppressive drugs. However, an antagonism was noted for two *R. oryzae* isolates for the combination of itraconazole and rapamycin and for one *M. circinelloides* isolate for the combination of ravuconazole and rapamycin. An equivalent (less than 10% difference) or a higher percentage of synergy was obtained when alternative MIC inhibition endpoints were used (data not shown).

By agar diffusion tests, decreases of  $\geq 2$  dilutions of the MIC of the antifungal suggesting synergy were observed for 50% of the isolates for the combination of cyclosporine and either amphotericin B or posaconazole (examples are presented in Fig. 1 and 2).

Antifungal combinations showed promising results against zygomycetes either in vitro or in animal models (6, 9, 10, 13, 28, 31), and some combinations between antifungal and nonanti-

fungal drugs have also been evaluated (1, 6, 35). A recent study, however, has shown that the combination of posaconazole and liposomal amphotericin B was not synergistic in murine models of zygomycosis (32).

Because calcineurin inhibitors are associated with improved outcome for SOT patients with some fungal infections such as cryptococcosis (15, 30), we decided to evaluate combinations between antifungals and immunosuppressive drugs against zygomycetes. We found that immunosuppressive drugs alone had antifungal activity against zygomycetes with variable levels of activity depending on the species and on the drug and that combinations of amphotericin B or posaconazole with immunosuppressive drugs could exhibit synergistic interactions. In particular, the synergy was observed with cyclosporine, rapamycin, and, to a lesser extent, tacrolimus. It could be of interest to test a wider panel of zygomycetes including other pathogenic species, such as *Cunninghamella*.

These results extend previous findings with other fungi such as *Cryptococcus neoformans* (7, 15, 30), *Candida albicans* (5, 17–19, 21, 26, 37, 38), and *Aspergillus fumigatus* (16, 33, 34). More recently, combinations between posaconazole and calcineurin inhibitors evaluated against zygomycetes showed indifference to synergistic interactions (22, 23). Given the recent demonstration of the limited in vivo activity of caspofungin against infection due to *R. oryzae* and the inhibition of glucan synthase activity (12) and clinical reports of synergy with amphotericin B in animal models (13) and human (29), testing the combination of echinocandins with immunosuppressive drugs should be investigated. Moreover, the combined inhibition of Fks1/2 and homeostatic cell wall stress responses in this fungi by the calcineurin-echinocandin combination would be phar-

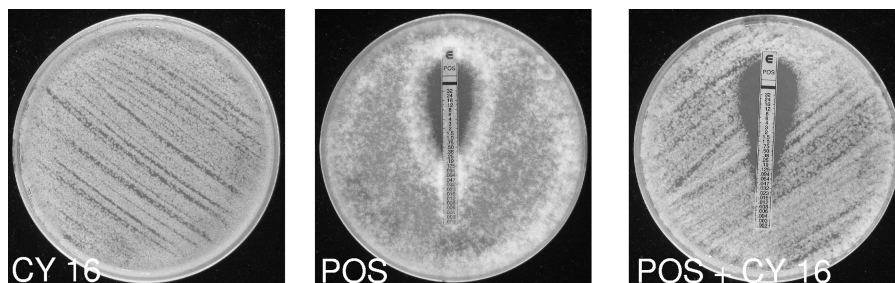


FIG. 2. In vitro interaction of posaconazole (POS) with cyclosporine (CY 16) against an isolate of *Mycocladius corymbiferus* (IP 1279.81) tested by an agar diffusion method. Cyclosporine was incorporated in the agar. The posaconazole MIC was determined by Etest in the absence or presence of cyclosporine at a concentration of 16  $\mu\text{g/ml}$ .

macologically sound. Similarly, it could be of interest to test if positive interactions could be observed if cells are exposed to H<sub>2</sub>O<sub>2</sub>, cycloheximide, or other nonspecific stress molecules.

In summary, we showed that calcineurin inhibitors and rapamycin could enhance the in vitro activity of amphotericin B and posaconazole against zygomycetes. These in vitro results warrant further animal experiments.

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