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Short Communication

The Effects of an Hsp90 Inhibitor on the Paradoxical Effect

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SUMMARY: It is important to conserve the effectiveness of antifungal agents because the options for currently available agents are limited. Although echinocandins, which have been developed in recent decades, are highly active against a broad spectrum of fungi, one concern is their reduced activity against *Candida albicans* at high drug concentrations, which is known as the paradoxical effect. To date, resistance related to the paradoxical effect has not been reported in clinical situations, but some in vivo data suggest that the paradoxical effect potentiates the emergence of resistance. It is valuable to investigate the underlying mechanisms of as well as strategies against this paradoxical resistance. Previous reports imply that the paradoxical effect might be related to stress responses. In this study, we report that radicicol, a heat shock protein 90 (Hsp90) inhibitor, reduces the paradoxical effect of micafungin. We also confirm that radicicol reduces the tolerance to voriconazole, one of the new azoles, which is consistent with a previous report. Our results may therefore imply that common stress responses might exist in the paradoxical resistance to micafungin and also the tolerance to voriconazole, and may suggest that inhibiting Hsp90-related stress responses could help to avoid potential resistances.

The emergence of resistance is one of the problems in intractable infections such as fungal infections. Because the options for currently available antifungal agents are still limited, it is important to maintain the effectiveness of these agents against fungi as long as possible (1,2). Though echinocandins have been developed in recent decades and are highly active against a broad spectrum of fungi, including *Candida* and *Aspergillus* spp., there are concerns regarding their reduced activity at high drug concentrations, which is known as the paradoxical effect (PE) (3). To date, the PE has been linked to upregulation of homeostatic cell wall stress responses such as calcineurin (4,5). Since heat shock protein 90 (Hsp90) is one of the key stress response components, we investigated whether an inhibitor of Hsp90 could reduce the PE of *Candida albicans*.

SC5314, a standard *C. albicans* strains from our collection, was used in this study (6). Micafungin (MFG), one of the echinocandins, and voriconazole (VRC), one of the new azoles, were kindly provided from Astellas Pharma Inc. (Tokyo, Japan) and Pfizer Japan Inc. (Tokyo, Japan), respectively. Radicicol (Rad, Hsp90 inhibitor; MIC = 8 μM) was purchased from Sigma-Aldrich (St. Louis, Mo., USA). MFG was dissolved in distilled water, and VRC and Rad were dissolved in dimethylsulfoxide (DMSO) for stock solutions, and they were stored at −20°C before use. We used yeast nitrogen base medium (YNB) (Difco Laboratories, Detroit, Mich., USA) instead of RPMI medium, which is recommended as a standard medium by the Clinical and Laboratory Standards Institute (CLSI) because growth is slow in RPMI, making it difficult to detect paradoxical growth.

After overnight preculture in YNB supplemented with 2% glucose, approximately 2×10^3 cells were inoculated in each well of 96-well plates. Each well contained 200 μl of YNB with the indicated concentrations of MFG or VRC; without Rad or with 1 μM Rad. The maximum final DMSO concentration (0.1%) was included in assays performed in the absence of Rad to control for any solvent effects. After a 24-h incubation period, the cell growth was compared using an XTT assay. Though the XTT assay is not usually used for examining planktonic cell growth, this assay is useful for detecting small amounts of cell growth such as that occurring with paradoxical growth because it is more sensitive than simple optical density measurements. The method was slightly modified from that previously described (7). In brief, the plates were centrifuged to spin down the organisms, the medium was removed, and 200 μl of PBS containing 50 μg/ml of XTT (Sigma-Aldrich) and 4 μM menadione (Sigma-Aldrich) was added to each well. After a 1-h incubation period, the absorbance at 490 nm/630 nm was measured by plate reader and values were normalized to the control (neither an antifungal agent nor Rad) as the relative cell growth. Each condition was quadruplicated. *P* values were calculated using the Student's unpaired *t* test.

As previously described for the PE, MFG severely inhibited cell growth to less than 1% at 0.031 and 0.063 μg/ml, and higher concentrations of MFG were less effective (Fig. 1A). The addition of 1 μM Rad, which alone did not alter growth, reduced the paradoxical cell growth at high concentrations of MFG. We confirmed that cyclosporin A, which is a calcineurin inhibitor, also attenuated the PE, as previously described (4) (data not shown). These results are consistent with the results from previous studies showing the relationship between the PE and stress-related cell integrity pathways (3-5,8,9). Rad also reduced the tolerance to VRC, as previously noted (Fig. 1B) (10). These results may suggest that the paradoxical resistance to MFG depends on the same stress responses as the tolerance to VRC.

Cowen et al. first reported that an Hsp90 inhibitor did not alter the sensitivity to caspofungin, an echinocandin, but they also recently reported that an Hsp90 inhibitor enhanced the

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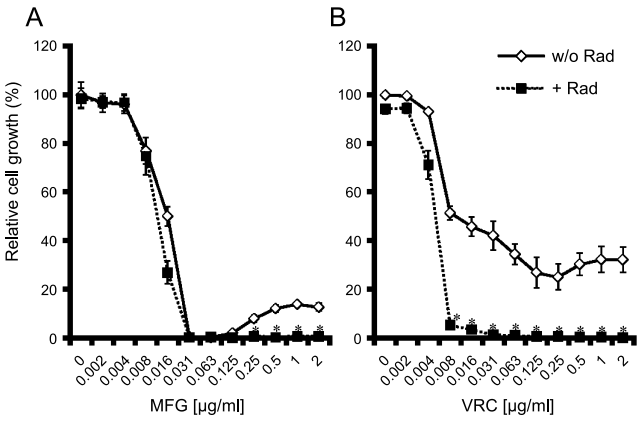

Fig. 1. Relative cell growth at various concentrations of MFG (A) or VRC (B); when used without Rad (w/o Rad) or with 1 μM Rad (+ Rad). All values are as a percentage of cell growth (XTT activity) in a control with neither antifungal agent nor Rad.
*$P < 0.01$ compared to corresponding value without Rad.
MFG, micafungin; VRC, voriconazole; Rad, radicicol.

fungicidal effects of MFG (10,11). Their latter finding might be related to our results.

It remains controversial whether the PE phenotype is clinically important. Only a mutation of *fks1*, which is a target of echinocandins, has been proven to cause clinical failure with echinocandins to date, and it has been reported that the PE is not due to the *fks1* mutation (9,12,13). However, some studies have found the PE in vivo as well as in vitro, and accordingly it is not deniable that the PE potentiates the threat of clinically relevant resistance (14-17). Therefore, inhibiting the PE may be important for avoiding the emergence of resistance, and regulating stress responses could be useful for inhibiting the PE.

In conclusion, the present findings regarding the PE and the development of resistance in antifungal agents could be valuable. It has never before been reported that an Hsp90 inhibitor reduces the PE, and this finding may suggest that inhibiting Hsp90-related stress responses could help to avoid potential resistances.

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